Hypoxie-vermittelte Mechanismen der Tumorprogression am Modell des Zervixkarzinoms

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Abstract: Hypoxie spielt eine wichtige Rolle bei einer Vielzahl von physiologischen und pathophysiologischen Vorgängen. Im Verlauf des letzten Jahrzehnts konnte für das Zervixkarzinom sowie für andere solide Tumoren die klinische Bedeutung der Tumorhypoxie für die Prognose der Patienten nachgewiesen werden. Dabei vermittelt die Hypoxie offenbar ihre Effekte einerseits über die Anpassung der Genexpression, was hauptsächlich über den Transkriptionsfaktor HIF-1 (hypoxie-induzierbarer Faktor-1) erreicht wird. Andererseits übt die Hypoxie einen Selektionsdruck auf die Tumorzellen aus, was letztlich zu einer genetischen Fixierung des „hypoxischen Phänotyps“ auch unter nicht-hypoxischen Bedingungen führen und damit das biologische und klinische Verhalten des Tumors nachhaltig beeinflussen kann. Ziel der vorliegenden Arbeit war es, molekulare Mechanismen zu identifizieren, die dem klinisch beobachteten aggressiveren Phänotyp hypoxischer Tumoren zugrunde liegen. Einerseits wurden hierzu In-vitro-Untersuchungen zur Identifizierung hypoxie-induzierter Gene durchgeführt. Andererseits wurde die Expression wichtiger hypoxie-induzierter Genprodukte im Zervixkarzinom untersucht und ihr Verhältnis zum aktuellen Grad der intratumoralen Hypoxie charakterisiert. Des Weiteren wurden molekulare Mechanismen der klinisch beobachteten Apoptoseresistenz - als wesentlicher Folge der hypoxie-gesteuerten Selektion - untersucht.

Hypoxie-vermittelte Mechanismen der Tumorprogression am Modell des Zervixkarzinoms

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Zusammenfassung

Hypoxie spielt eine wichtige Rolle bei einer Vielzahl von physiologischen und pathophysiologischen Vorgängen. Im Verlauf des letzten Jahrzehnts konnte für das Zervixkarzinom sowie für andere solide Tumoren die klinische Bedeutung der Tumorhypoxie für die Prognose der Patienten nachgewiesen werden. Dabei vermittelt die Hypoxie offenbar ihre Effekte einerseits über die Anpassung der Genexpression, was hauptsächlich über den Transkriptionsfaktor HIF-1 (hypoxie-induzierbarer Faktor-1) erreicht wird. Andererseits übt die Hypoxie einen Selektionsdruck auf die Tumorzellen aus, was letztlich zu einer genetischen Fixierung des „hypoxischen Phänotyps“ auch unter nicht-hypoxischen Bedingungen führen und damit das biologische und klinische Verhalten des Tumors nachhaltig beeinflussen kann.

Ziel der vorliegenden Arbeit war es, molekulare Mechanismen zu identifizieren, die dem klinisch beobachteten aggressiveren Phänotyp hypoxischer Tumoren zugrunde liegen. Einerseits wurden hierzu *In-vitro*-Untersuchungen zur Identifizierung hypoxie-induzierter Gene durchgeführt. Andererseits wurde die Expression wichtiger hypoxie-induzierter Genprodukte im Zervixkarzinom untersucht und ihr Verhältnis zum aktuellen Grad der intratumoralen Hypoxie charakterisiert. Des Weiteren wurden molekulare Mechanismen der klinisch beobachteten Apoptoseresistenz - als wesentlicher Folge der hypoxie-gesteuerten Selektion - untersucht.

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1. Einleitung

1.1 Allgemeines

Unter Hypoxie versteht man ein Absinken des Sauerstoffpartialdrucks (pO_2) unter eine kritische Schwelle, ab der es zu einer Einschränkung oder gar zu einem Verlust biologischer Funktionen in Organen, Geweben oder Zellen kommt (1). Hypoxie spielt eine wesentliche Rolle bei einer Vielzahl von physiologischen als auch pathophysiologischen Vorgängen. Die Fähigkeit von Zellen, auf eine hypoxische Umgebung zu reagieren, ist notwendig, um verminderte Sauerstoffkonzentrationen zu kompensieren, wie sie zum Beispiel in der Embryonalentwicklung (2) aber auch bei Wundheilungsvorgängen (3) auftreten können. Das Vorkommen von Hypoxie in soliden Tumoren war erstmals von Thomlinson und Gray vor mehr als fünfzig Jahren beschrieben worden (4).

Im Verlauf des letzten Jahrzehnts konnten verschiedene Arbeitsgruppen die klinische Bedeutung der intratumoralen Hypoxie für die Prognose verschiedener solider Tumoren zeigen, z.B. für das Zervixkarzinom (5), Kopf-Hals-Tumoren (6) und das Weichteilsarkom (7). So konnten Höckel et al. bereits 1996 nachweisen, dass hypoxische Zervixkarzinome eine ungünstigere Prognose für die Patientinnen bedeuten und zwar sowohl bei operativer wie auch radiotherapeutischer Behandlung (5). Das schlechtere Ansprechen hypoxischer Tumoren auf eine Strahlentherapie lässt sich unmittelbar aus der Wirkungsweise ionisierender Strahlen erklären (8). Dagegen war die Beobachtung, dass auch chirurgisch behandelte hypoxische Karzinome eine schlechtere Prognose hatten, zunächst überraschend. Dieser Befund wies darauf hin, dass zwischen hypoxischen und nicht-hypoxischen Tumorzellen fundamentale biologische Unterschiede bestehen müssen, die zu einem aggressiveren klinischen Verhalten hypoxischer Tumoren führen.

Verschiedene Faktoren sind am Entstehen einer chronischen bzw. akuten Tumorhypoxie beteiligt: Einerseits kommt es in rasch wachsenden soliden Tumoren zu einem erhöhten Sauerstoffverbrauch, der bald das Sauerstoffangebot übersteigt. Die resultierende intratumorale (chronische) Hypoxie ergibt sich aus erhöhten Diffusionsstrecken zwischen den vorhandenen Blutgefäßen und den sauerstoffverbrauchenden Tumorzellen und kann durch eine tumor- oder therapieassoziierte Anämie eventuell noch verstärkt werden (9). Andererseits kommt es zu kurzfristigen Schwankungen der intratumoralen Oxygenierung, die durch den Wechsel von Thrombosen und Fibrinolysen in pathologischen Tumorgefäßen bedingt sind (akute Hypoxie und Reoxygenierung, (10)).

Ein wesentlicher Mechanismus, über den die Hypoxie ihre Effekte vermittelt, ist die differentiell regulierte Genexpression. Dabei stellt der Hypoxia-Inducible Factor-1 (HIF-1) den wichtigsten hypoxie-induzierbaren Transkriptionsfaktor dar (11). Dieser Transkriptionsfaktor induziert die Expression einer Vielzahl von Genen, die aufgrund der Funktion ihrer

Proteinprodukte im Wesentlichen vier verschiedenen funktionellen Gruppen zugeordnet werden können: dem Metabolismus, der Angiogenese, der Invasion/Metastasierung und der Apoptose (12) (Abbildung 1). Es wird angenommen, dass die Überexpression dieser Gene (bzw. Genprodukte) mitverantwortlich ist dafür, dass Tumorzellen aggressiver werden und eine Resistenz gegenüber verschiedenen Therapiemodalitäten erwerben können.

Zusätzlich zu den hypoxie-bedingten Veränderungen der Genexpression kann die Tumorhypoxie zu einer genetischen Instabilität führen, indem sie Punktmutationen, Genamplifikationen und chromosomale Rearrangements induziert bzw. ermöglicht (13), und damit die Zahl genetischer Varianten innerhalb des Tumors erhöht. Des Weiteren übt die Hypoxie als Apoptosestimulus einen starken Selektionsdruck auf die Tumorzellen aus. Auf diese Weise können sich solche malignen Zellen klonal ausbreiten, die unter der Hypoxie bestimmte Eigenschaften erworben haben, die einen Überlebensvorteil im hypoxischen Mikromilieu bedeuten (14). Über die Initiierung von Selektionsprozessen haben auch Zellen einen Selektionsvorteil, die bereits vor dem hypoxischen Einfluss bestimmte Mutationen oder epigenetische Veränderungen angenommen haben. Die hypoxie-gesteuerte Selektion von genetischen Varianten kann letztlich zu einer genetischen Fixierung des „hypoxischen Phänotyps“ auch unter nicht-hypoxischen Bedingungen führen und damit das biologische und klinische Verhalten des Tumors nachhaltig beeinflussen (1).

Ziel aller hier zusammengefassten Arbeiten war es, zum einen hypoxie-induzierte Veränderungen der Genexpression *in vitro* zu untersuchen. Zum anderen sollte der Zusammenhang zwischen Hypoxiemarkern und der intratumoralen Oxygenierung in klinischen Zervixkarzinomen analysiert und ihr Einfluss auf die maligne Progression charakterisiert werden (Abbildung 1). Des Weiteren sollten die molekularen Mechanismen der klinisch beobachteten erworbenen Apoptoseresistenz - als wesentlicher Folge der hypoxie-gesteuerten Selektion - untersucht werden.

1.2 Hypoxie-induzierte Transkriptionsfaktoren

HIF-1 wurde erstmals im Zusammenhang mit der Erforschung der Mechanismen beschrieben, die zur Induktion des Erythropoietin-Gens führen (15, 16). Es handelt sich um einen heterodimeren Transkriptionsfaktor, der zur Familie der basic helix-loop-helix-PAS (PER/ARNT/SIM)-Transkriptionsfaktoren gehört. Er besteht aus der hypoxie-abhängigen HIF-1α-Untereinheit und der konstitutiv exprimierten HIF-1ß-Untereinheit. Unter hypoxischen Bedingungen wird das normalerweise labile HIF-1α stabilisiert, was zu seiner raschen intrazellulären Anhäufung führt. Nach der Translokalisation in den Zellkern kommt es zur Heterodimerisierung mit HIF-1ß. Der Komplex bindet anschließend an spezifische DNA-Sequenzen innerhalb sogenannter hypoxie-responsiver Elemente (HRE), was zur Transkription der entsprechenden Targetgene führt (11). Im Gegensatz dazu kommt es unter

normoxischen Bedingungen zu einem raschen Abbau der HIF-1α-Untereinheit über das Ubiquitin-Proteasom-System, vermittelt über das von-Hippel-Lindau-Protein (VHL). VHL kann nur an HIF-1α binden, wenn bestimmte sauerstoffabhängige enzymatische Modifikationen vorliegen (17).

Allerdings kann HIF-1α auch durch hypoxie-unabhängige, tumor-spezifische Faktoren stabilisiert werden (18). Dazu gehören diverse Wachstumsfaktoren, Zytokine, die Aktivierung bestimmter Onkogene (z.B. src, ras) und der Verlust von Tumorsuppressorgenen (PTEN, VHL, p53). Neben HIF-1 existieren noch weitere hypoxie-induzierbare Transkriptionsfaktoren, z.B. NF-kB, AP-1, und early growth response factor-1. Jedoch handelt es sich bei diesen um stress-responsive Transkriptionsfaktoren allgemeinerer Art, deren Reaktion auf Hypoxie weit weniger spezifisch ist als die von HIF-1.

Zusätzlich zur hypoxie-induzierten Transkription von Targetgenen können auch posttranskriptionale Mechanismen wie die Stabilisierung von messenger RNA (mRNA) zur Überexpression bestimmter Genprodukte unter Hypoxie führen.

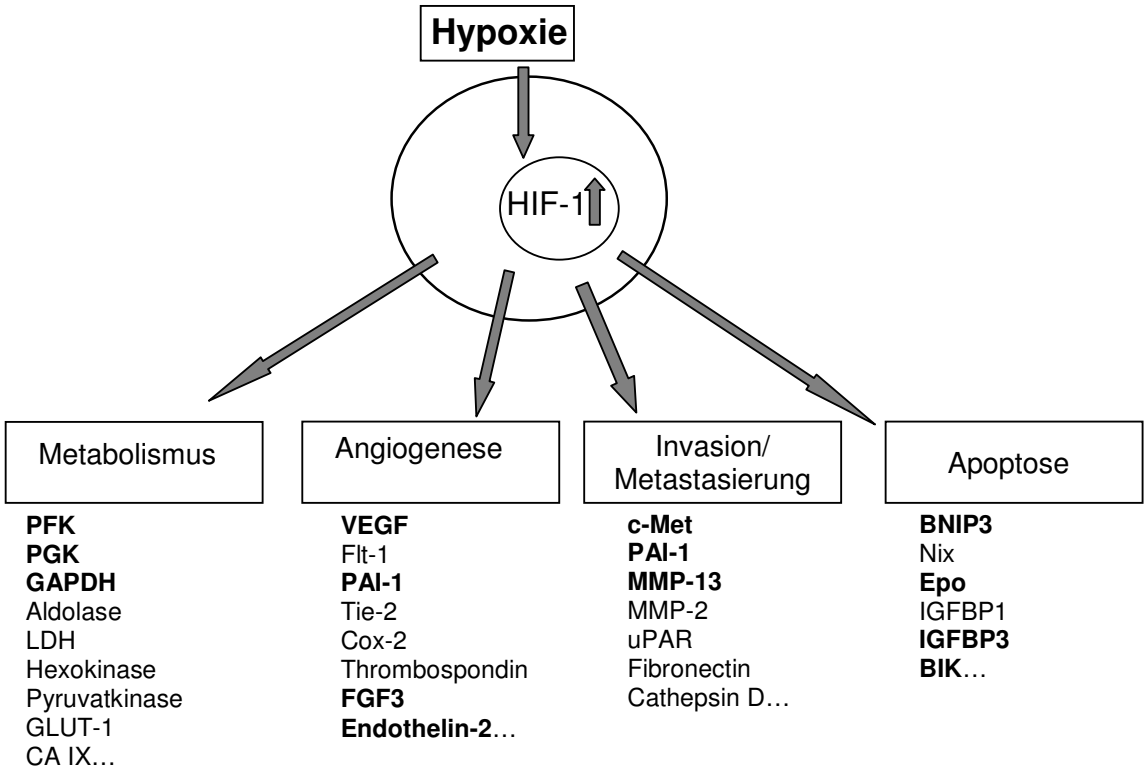

Abbildung 1: Hypoxie-induzierte Genexpression (Auswahl). Fettgedruckt sind Genprodukte, die im Rahmen der Arbeit identifiziert und/oder charakterisiert wurden (Auswahl).

1.3 Hypoxie-induzierte Veränderungen in Tumorzellen

Basierend auf der Auswertung von Expressionsanalysen wird davon ausgegangen, dass ca. 1,5% des Genoms unter hypoxischen Bedingungen auf transkriptionaler Ebene aktiviert werden (19). Hypoxie-regulierte Gene sind in verschiedenste biologische Prozesse eingebunden und lassen sich in funktionelle Kategorien einteilen. So sind hypoxie-induzierte Gene z.B. an der Regulation von Stoffwechselfunktionen, der Angiogenese, von Metastasierungsvorgängen und der Apoptose beteiligt. Mit der Beeinflussung dieser vielfältigen Funktionen gelingt es den Tumorzellen, sich an die ungünstigen Bedingungen eines hypoxischen Mikromilieus zu adaptieren, was ihnen nicht nur ihr Überleben ermöglicht, sondern sogar die Voraussetzungen dafür schafft, zu proliferieren und/oder das unwirtliche Mikromilieu zu verlassen.

1.3.1 Metabolismus

Um in der Hypoxie Energie zu generieren, müssen Zellen vom aeroben Zitratzyklus auf die anaerobe Glykolyse umschalten. Da die Glykolyse pro Glukosemolekül wesentlich weniger Energie bereitstellt als der Zitratzyklus (2 ATP-Moleküle vs. 38 ATP-Moleküle), muss der Gesamtglukoseverbrauch gesteigert werden. HIF-1 aktiviert eine Vielzahl glykolytischer Enzyme, z.B. Phosphofruktokinase, Hexokinase, Lactatdehydrogenase, Pyruvatkinase und Aldolase. Auch die Expression der Glukosetransporter Glut-1 und Glut-3 wird gesteigert, um den intrazellulären Glukosenachschub zu gewährleisten (11). Der anaerobe Glukoseumsatz führt zur Anhäufung von Laktat, woraus eine intra- und extrazelluläre Azidose resultiert. Der ebenso über HIF-1 vermittelte Anstieg der Carboanhydrasen IX und XII trägt zur Regulation der sauren Umgebung bei (12).

1.3.2 Angiogenese

Um sich anzupassen, kommt es in hypoxischen Geweben zu einer Hochregulierung verschiedener proangiogener Faktoren, während antiangiogene Faktoren reprimiert werden. Das koordinierte Zusammenspiel einer Vielzahl angiogener Proteinprodukte ist Voraussetzung für die Gefäßneubildung, wobei dem HIF-1-induzierbaren Vascular Endothelial Growth Factor (VEGF) eine Schlüsselrolle zukommt. Weitere Gene, die bei der Angiogenese in einem hypoxischen Mikromilieu von Bedeutung sind, umfassen den VEGF Receptor-1 (Flt-1), Plasminogen Activator Inhibitor-1 (PAI-1), Angiopoietin-2, Tie-2, Cyclooxygenase (COX)-1, COX-2, iNOS, Adrenomedullin, FGF-3, Monocyte Chemotactic Protein-1, Osteopontin, Histone Deacetylase, TGFα, ß1, ß3 und Hepatocyte Growth Factor (HGF; (12)). Außerdem führt Hypoxie zur Hemmung von antiangiogenen Faktoren, wie z.B. Thrombospondin I und II (19). Die resultierenden Tumorgefäße weisen jedoch eine pathologische und chaotische Gefäßarchitektur auf, die das hypoxische Mikromilieu und

damit die hypoxische Genaktivierung aufrechterhalten können (20, 21). Neben der Induktion von Angiogenesefaktoren kann HIF-1 auch Gene aktivieren, die eine verstärkte Sauerstoffversorgung in den peripheren Geweben unterstützen; die Transaktivierung von Erythropoietin, Transferrin, Transferrin-Rezeptor und Hämoxygenase ist z.B. bei der Anhebung des Hämoglobinlevels als Reaktion auf Hypoxie beteiligt.

1.3.3 Invasion und Metastasierung

Hypoxie beinflusst die Invasivität und das Metastasierungspotential von Tumoren (7). *In vitro*-Untersuchungen zeigten, dass Hypoxie in Kombination mit Reoxygenierung zu einem deutlichen, wenn auch vorübergehenden, Anstieg des metastatischen Potentials von Maustumorzellen führte (22). Zwischenzeitlich wurden verschiedene hypoxie-regulierte Gene identifiziert, die in Invasions- und Metastasierungsprozesse einbezogen sind. So konnten Pennacchietti et al. zeigen, dass Hypoxie zur Transaktivierung des c-Met-Protoonkogens führt (23). c-Met ist der Rezeptor für HGF und sensibilisiert auf diesem Wege hypoxische Tumorzellen für die pro-invasiven Effekte von HGF. Die c-Met-Aktivierung führt zu Zellproliferation sowie Zelldissoziation, erhöht die Zellmotilität, erleichtert die Invasion in die umgebende Matrix und ermöglicht Formänderungen der betreffenden Zellen (sog. branching morphogenesis) (24), was klinisch zu einer Tumormetastasierung beitragen könnte.

Andere potentielle Gene bzw. Genprodukte, die das Metastasierungpotential hypoxischer Tumoren erhöhen könnten, sind PAI-1 und Urokinase-type Plasminogen Activator Receptor (uPAR; (25, 26). Kürzlich wurden auch einige extrazelluläre Matrix-bzw. Adhäsionsmoleküle, wie Cathepsin D, Fibronectin und Matrix Metalloproteinase-2, als hypoxie-induzierbar identifiziert, womit ein weiterer möglicher molekularer Mechanismus für den invasiven / metastatischen Phänotyp in einem hypoxischen Milieu aufgedeckt wurde (27).

1.3.4 Apoptose

Der Zusammenhang zwischen der Hypoxie von Zellen bzw. Geweben und der Induktion der Apoptose ist komplex. Einerseits ist Hypoxie ein anerkannter Apoptosestimulus, anderseits kann unter Hypoxie offenbar eine Selektion apoptoseresistenter Tumorzellen erfolgen.

Eine moderate Hypoxie mit einem Sauerstoffanteil von ≥2% ist in der Regel noch nicht als Apoptosestimulus ausreichend (28). Unter Bedingungen der schweren Hypoxie bzw. Anoxie jedoch kommt es zur Auslösung verschiedener zellulärer Ereignisse, die letztlich im programmierten Zelltod resultieren. Besteht zusätzlich zur Hypoxie/Anoxie auch eine deutliche Verminderung der Energieversorgung mit ATP, wird anstelle der Apoptose der Zelltod über Nekrose ausgelöst (29).

Ein Mechanismus der hypoxie-induzierten Apoptose besteht in der direkten Hemmung der mitochondrialen Atmungskette. Der Sauerstoffmangel hemmt den Protonentransport und senkt damit das mitochondriale Membranpotential. Die daraus folgende Verminderung des Energieträgers ATP aktiviert die proapoptotischen Moleküle Bax und Bak, was zur Cytochrom-C–Ausschüttung aus den Mitochondrien ins Zytosol mit Aktivierung der nachgeschalteten Apoptosekaskade führt (30). Während einer nachfolgenden Reoxygenierung kann ausserdem die Bildung von Sauerstoffradikalen („reactive oxygen species“, ROS) einen weiteren Beitrag zur hypoxie-induzierten Apoptose leisten (30).

Auch der Transkriptionsfaktor HIF-1 spielt eine wichtige Rolle bei der hypoxie-induzierten Apoptose. Einerseits stabilisiert er das p53-Tumorsuppressor-Genprodukt, welches dann das proapoptotische Bax aktivieren kann (31). Andererseits werden durch HIF-1 die proapoptotischen Gene BNIP3 und NIX hochreguliert (32). Eine Überexpression von BNIP3 wurde unter hypoxischen Bedingungen in verschiedenen Karzinomzelllinien nachgewiesen (33). Obwohl BNIP3 und NIX unter normoxischen Bedingungen Apoptose auslösen (34) sind unter hypoxischen Bedingungen möglicherweise zusätzliche Faktoren zur Apoptoseauslösung nötig (z.B. Azidose; (35)).

Neben der Beeinflussung von proapoptotischen Molekülen haben Zellen auch die Möglichkeit, antiapoptotische Signale als Reaktion auf die hypoxische Umwelt zu regulieren. So konnte gezeigt werden, dass das Inhibitor-of-Apoptosis-Protein-2 (IAP-2) hypoxie-induzierbar ist und eine Apoptose unter hypoxischen Bedingungen unterdrücken kann (36).

Aufgrund ihrer proapoptotischen Effekte kann eine anhaltende Hypoxie offenbar auch zu einer Apoptoseresistenz führen. Hypoxie tritt schon frühzeitig in wachsenden Tumoren auf und übt damit auch einen frühen Selektionsdruck auf die Tumorzellen aus, der solchen Tumorklonen einen Überlebensvorteil bietet, die bereits gegen eine hypoxie-induzierte Apoptose resistent sind. Weiterhin kann durch die Einwirkung einer prolongierten Hypoxie (eventuell auch in Kombination mit Reoxygenierungsperioden) die genetische Instabilität zunehmen, wodurch Varianten entstehen, die die Hypoxie überleben können (13). Diese erworbene hypoxie-induzierte Apoptoseresistenz kann offenbar ihrerseits zu einem klinisch aggressiveren Tumorphänotyp führen. In einer klinischen Studie zum Zervixkarzinom konnten Höckel et al. zeigen, dass Patientinnen, deren Karzinome trotz Hypoxie eine niedrige Apoptoserate aufwiesen, eine signifikant schlechtere Prognose hatten im Vergleich zu den Patientinnen mit hypoxischen Tumoren und hoher Apoptoserate bzw. mit normoxischen Tumoren (37). Allerdings sind die Mechanismen, die zu dieser unter Hypoxie erworbenen Apoptoseresistenz führen, bisher weitgehend unklar. *In vitro* bzw. in Mausmodellen wurde eine mögliche Rolle für p53 und für Apaf-1 aufgezeigt (38, 39).

1.4 Das Zervixkarzinom als Modell für hypoxische Tumoren

Aufgrund seiner Lokalisation ist das Zervixkarzinom einer invasiven Messung des intratumoralen Sauerstoffdruckes (pO_2) mit der Eppendorf-Elektrode besonders gut zugänglich und ermöglicht eine unkomplizierte Durchführung der Stanzbiopsien, die die Gewinnung von Gewebe entlang des Messkanals erlauben. Ein weiterer Vorteil ist, dass eine Messung sowohl in frühen wie auch fortgeschrittenen Tumorstadien möglich ist.

Zudem ist das Zervixkarzinom der Tumor, der als Modell für die Validierung der Eppendorf-Technik diente. Die Eppendorf-Elektrode ist eine polarografische Nadelelektrode, die unter Verwendung eines computerisierten O_2-Sensors die systematische Evaluierung der Gewebeoxygenierung erlaubt (40). Mithilfe dieser Technik konnten viele Informationen über den Oxygenierungsgrad klinischer Tumoren gewonnen werden und trotz der Invasivität der Messung stellt die Eppendorf-Elektrode nach wie vor den Goldstandard zur Bestimmung des intratumoralen pO_2 dar (41-43).

Das Zervixkarzinom ist die Tumorart, für die die klinische Bedeutung der intratumoralen Hypoxie für die Prognose am besten durch klinische Studien belegt ist. Hypoxische Zervixkarzinome sind, unabhängig von der Therapiemodalität, mit einer ungünstigeren Prognose für die Patientinnen assoziiert (5). Besonders die hypoxischen Karzinome mit einer niedrigen Apoptoserate waren mit einem signifikant schlechteren Überleben verbunden (37). Auch konnte gezeigt werden, dass Rezidive von Zervixkarzinomen signifikant schlechter oxygeniert waren als die entsprechenden Primärtumoren (44).

1.5 Methoden zur Tumorhypoxie-Messung

Neben der Eppendorf-Elektrode als Goldstandard existieren noch weitere invasive und nicht-invasive Methoden zur Bestimmung der intratumoralen Oxygenierung. Tabelle 1 fasst verschiedene physikalische und chemische Verfahren der invasiven und nicht-invasiven, direkten und indirekten pO_2-Messung zusammen. Welche Methode für einen bestimmten experimentellen Ansatz oder eine definierte klinische Fragestellung geeignet ist, hängt von verschiedenen Faktoren ab, so z.B. von der Tumorlokalisation und der erforderlichen Auflösung.

Tabelle 1: Methoden für die Messung der Tumorhypoxie, nach Höckel und Vaupel (1)

1) Invasive Mikrosensortechniken • Polarografische O_2-Sensoren • Lumineszenz-basierte optische Sensoren
2) Paramagnetische Elektronen-Resonanz-Oxymetrie
3) Techniken für die intravaskuläre O_2-Messung • Kryospektrophotometrie • Infrarot-Spektroskopie • Phosphoreszenz-Imaging
4) Magnetresonanzspektroskopie und Imaging-Techniken • ^{1}H- Magnetresonanzspektroskopie • ^{19}F-Magnetische-Resonanz-Relaxometrie
5) Nicht-invasive Detektion von Sensitizer-Addukten • ^{18}F-Fluoromisonidazol (PET) • ^{123}I-Iodoazomycin-Arabinosid (SPECT)
6) Invasive Techniken unter Nutzung metabolisch aktivierter chemischer Marker • Misonidazol • Pimonidazol • Etanidazol • Nitroimidazol-Theophyllin

2. Zusammenfassung der eigenen Arbeiten

2.1 Einführung in die Arbeiten 2.1.1 – 2.1.4

Einen wesentlichen Mechanismus, über den die Hypoxie ihre Effekte vermittelt, stellt die differenzierte Anpassung der Genexpression dar. Die Induktion von Genen wird über hypoxie-induzierte Transkriptionsfaktoren vermittelt, wobei HIF-1 die wichtigste Rolle zukommt. Mit der fein abgestimmten Regulierung des Transkriptoms gelingt es der Zelle, sich an die widrigen Bedingungen eines hypoxischen Mikromilieus anzupassen.

Darüber hinaus wird angenommen, dass wesentliche Teilaspekte des klinisch beobachteten aggressiven Tumorphänotyps (z.B. Invasion und Metastasierung) einigen der hypoxie-induzierten Gene zuzuschreiben sind. Die hier zusammengefassten Arbeiten beschäftigen sich mit der Identifikation von hypoxie-induzierten Genen *in vitro*. Dabei wurden Karzinomzellen unter Bedingungen einer einmaligen bis zu 48 Stunden dauernden Hypoxie mit Hilfe von DNA-Arrays, subtraktiver Hybridisierung und Northern Blots untersucht. In der Folge wurden sowohl neue Gene identifiziert, als auch bekannte Gene mit zuvor unbekannter Hypoxieabhängigkeit beschrieben. In der Arbeit 2.1.4 wird ein hypoxie-unabhängiger Mechanismus der HIF-1-vermittelten Genexpression charakterisiert.

2.1.1 Die Bedeutung des hypoxischen Mikromilieus für die Genexpression

Leo C, Giaccia AJ, Denko NC.
The hypoxic tumor microenvironment and gene expression.
Sem Radiat Oncol. 2004;14:207-14.

Solide Tumoren sind im Laufe ihrer Entwicklung einer ständigen Beeinflussung durch die Tumorumgebung ausgesetzt. Dabei entwickeln sie Mechanismen, die es ihnen ermöglichen, trotz widriger Bedingungen zu überleben und sogar zu proliferieren. Wesentlich hierfür ist ihre Fähigkeit zur koordinierten Anpassung der Genexpression, wobei Gene entweder induziert oder reprimiert werden können. Dieser Mechanismus erlaubt eine biologische Anpassung an die ungünstigen Bedingungen eines sauerstoffarmen Tumor-Mikromilieus. Die Tumorhypoxie stellt einen Stress dar, der in nahezu jedem soliden Tumor vorkommt und sowohl zur Tumorprogression als auch zur Therapieresistenz beitragen kann. Der konkrete Einfluss einzelner hypoxie-induzierter Gene einerseits sowie des gesamten hypoxie-responsiven Gen-Netzwerks andererseits ist Gegenstand intensiver Forschungsbemühungen. Ein besseres Verständnis der biologischen Funktionen von hypoxie-induzierten wie auch -reprimierten Genen im Tumorgewebe wird letztlich Wege zu einer besseren therapeutischen Beeinflussung hypoxischer Tumoren eröffnen.

In dieser Arbeit geben wir einen Überblick über ausgewählte hypoxie-induzierte Gene und diskutieren ihre Bedeutung für die Tumorphysiologie. Dabei wird dem Zusammenhang zwischen Tumorhypoxie und Hypoxiemarkern *in vivo* besondere Beachtung geschenkt.

2.1.2 Kandidatengene für die Entstehung des hypoxischen Tumorphänotyps

Koong AC, Denko NC, Hudson KM, **Schindler C**, Swiersz L, Koch C, Evans S, Ibrahim H, Le QT, Terris DJ, Giaccia AJ.
Candidate genes for the hypoxic tumor phenotype.
Cancer Res. 2000; 60(4):883-7.

Um hypoxie-induzierte Veränderungen der Genexpression auf transkriptionaler Ebene zu untersuchen, verwendeten wir DNA-Arrays. Zellen einer Plattenepithelkarzinom-Zelllinie (FaDu, Hypopharynxkarzinom) wurden entweder unter normoxischen Bedingungen oder in einer speziellen Hypoxiekammer (O_2<0,05%) kultiviert. Nach 6 h bzw. 18 h Hypoxie wurde RNA isoliert, die dann auf den DNA-Arrays analysiert und mit der RNA der normoxischen Zellen verglichen wurde. Auf diese Weise konnten wir eine Vielzahl hypoxie-induzierter Genen identifizieren, darunter Plasminogen Activator Inhibitor-1 (PAI-1), Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3), Endothelin-2, Low-Density Lipoprotein Receptor-Related Protein (LRP), BCL2-Interacting Killer (BIK), Migration-Inhibitory Factor (MIF), Matrix Metalloproteinase-13 (MMP-13), Fibroblast Growth Factor-3 (FGF-3), GADD45 und Vascular Endothelial Growth Factor (VEGF). Die Induzierbarkeit unter Hypoxie wurde für jedes einzelne Gen mittels Northern Blot-Analyse bestätigt und ihr zeitlicher Verlauf näher charakterisiert. Hierbei wurde neben den FaDu-Zellen auch die Zervixkarzinom-Zelllinie SiHa verwendet.

Das Verhalten von PAI-1 unter Hypoxie untersuchten wir noch detaillierter. Hintergrund hierfür ist die Tatsache, dass PAI-1 ein sezerniertes Protein ist und damit prinzipiell als (im Patientenserum nachweisbarer) molekularer Hypoxiemarker verwendet werden könnte. Weiterhin wurde bereits von verschiedenen Arbeitsgruppen gezeigt, dass die PAI-1-Proteinexpression in verschiedenen Tumoren signifikant mit einer schlechteren Prognose korrelierte (45-48). Wir konnten zeigen, dass die PAI-1-mRNA zwischen 2 h und 24 h Hypoxie graduell zunimmt, bevor es nach nur 2 h Reoxygenierung zu einem raschen Abbau kommt. Des Weiteren analysierten wir in dieser Arbeit die PAI-1-Serumlevel von Patienten mit Kopf-Hals-Tumoren und konnten zeigen, dass diese zum intratumoralen Oxygenierungsgrad der entsprechenden Tumoren, der zuvor mit der Eppendorf-Elektrode gemessen worden war, korrelierten. Damit stellt PAI-1 ein hypoxie-reguliertes sezerniertes Protein dar, welches – nach entsprechender weiterer Validierung - potentiell bei der Diagnosestellung, Prognoseabschätzung oder auch im Bereich der Nachsorge Verwendung finden könnte.

2.1.3 Beeinflussung der Genexpression in Zervixkarzinomzellen durch die Tumorumgebung

Denko N, **Schindler C**, Koong A, Laderoute K, Green C, Giaccia A.
Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment.
Clin Cancer Res. 2000; 6:480-7.

Um den Einfluss von hypoxischem Stress auf Zervixkarzinomzellen zu untersuchen, wurde in dieser Arbeit eine subtraktive Hybridisierungsmethode gewählt. Mit dieser „representational difference analysis“ wurden 12 hypoxie-induzierte Gene identifiziert. Darunter befanden sich die beiden zuvor nicht beschriebenen Gene HIG1 und HIG2 (HIG: Hypoxia Inducible Gene), drei als hypoxie-induziert bekannte Gene (Tissue Factor, GAPDH, Thioredoxin) und sieben bekannte Gene, deren Hypoxieabhängigkeit zuvor nicht beschrieben worden war (HNRNP(a1), ribosomales L7, Annexin V, Lipocortin 2, Ku70, PRPP synthase, Acetoacetyl-CoA Thiolase). Alle 12 Gene wurden einzeln im Northern Blot als hypoxie-induzierbar in verschiedenen Zervixkarzinom-Zelllinien bestätigt und der zeitliche Verlauf der Induktion charakterisiert.

Da HIG1 und HIG2 zuvor unbekannte Gene darstellten, analysierten wir diese weitergehend: Beide Gene sind sowohl durch Hypoxie als auch durch Glucoseentzug induzierbar. Des Weiteren zeigen Tumorxenografte, die von Zervixkarzinomzellen (C33a) abstammen, unter Hypoxie einen Anstieg der HIG1- und HIG2-mRNA-Expression. Wir überprüften auch, wo die HIG1- und HIG2-Proteine in Zellen exprimiert werden. Dazu wurden die Gen-Sequenzen mit Hämagglutinin-Tags (HA-Tags) versehen und die entstandenen Konstrukte in Zervixkarzinomzellen transfiziert. Das in den Zellen exprimierte Protein konnte dann mit einem Antikörper gegen das HA-Tag sichtbar gemacht werden. Auf diese Weise konnten wir zeigen, dass HIG1 mitochondrial exprimiert wird, während HIG2 mehr diffus im Zytoplasma nachgewiesen wurde.

Die hier neu beschriebenen Gene HIG1 und HIG2 stellen potentielle Kandidaten dar, die die Prognose von Patienten mit hypoxischen Tumoren beeinflussen könnten.

2.1.4 Der Verlust des Tumorsuppressorgens PTEN ermöglicht die HIF-1 vermittelte Genexpression

Zundel W, **Schindler C**, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ.
Loss of PTEN facilitates HIF-1-mediated gene expression.
Genes Dev. 2000; 14(4):391-6.

Das Tumorsuppressorgen PTEN ist in verschiedenen Tumoren häufig mutiert, so z.B. im Glioblastom, Endometriumkarzinom, Vulvakarzinom und Prostatakarzinom (49-52). PTEN spielt eine wesentliche Rolle bei der negativen Regulierung des PI(3)K-Akt-Survival-Pathways, der sowohl Anti-Apoptose, als auch Proliferation und Angiogenese vermittelt. Da über diesen Pathway auch der Transkriptionsfaktor HIF-1 stabilisiert werden kann, untersuchten wir in Glioblastom-Zelllinien, ob PTEN zur Regulierung von hypoxie-induzierter Genexpression beiträgt. Dazu verwendeten wir Glioblastom-Zelllinien, die kein funktionelles PTEN besitzen. In diese Zellen wurde mittels retroviraler Transfektion funktionelles PTEN eingeschleust. Anschliessend wurden die Zellen einer Hypoxie ausgesetzt und die Expression verschiedender hypoxie-regulierter Gene (VEGF, COX-1, PFK und PGK-1) im Northern Blot analysiert. Nur in den Zellen mit funktioneller PTEN-Expression kam es zur Blockierung der endogenen VEGF-, COX-1-, PGK-1 und PFK-Induktion unter Hypoxie. Dieses Ergebnis spricht für eine klare Beteiligung von PTEN an der Kontrolle der hypoxie-induzierten Genexpression.

Im Rahmen der besprochenen Arbeit wurde auch gezeigt, dass PTEN die durch Akt vermittelte HIF-1α-Stabilisierung abschwächte.

Zusammenfassend scheint der Verlust des Tumorsuppressors PTEN während der malignen Progression zur Tumorausbreitung beizutragen, indem die Akt-Aktivität und die HIF-1-vermittelte Genexpression dereguliert werden.

2.2 Einführung in die Arbeiten 2.2.1 – 2.2.3

In den letzten Jahren wurde für eine Vielzahl von Genen deren Hypoxie-Induzierbarkeit *in vitro* gezeigt. Die Relevanz dieser Befunde im Kontext eines klinischen Tumors und ihre Abhängigkeit vom intratumoralen pO_2 wurden hingegen kaum untersucht. (53, 54). Von den vielen *in vitro* hypoxie-induzierten Genprodukten konnte bisher nur für die Carboanhydrase IX (CA IX) und den Glukosetransporter Glut-1 eine Korrelation zur intratumoralen Oxygenierung *in vivo* gezeigt werden. So war die Glut-1-Expression in Zervixkarzinomen in Regionen intratumoraler Hypoxie erhöht, wobei die Hypoxie sowohl durch invasive pO_2-Messung mit der Eppendorf-Elektrode als auch durch Pimonidazolfärbung nachgewiesen wurde (55). Außerdem war ein Fehlen der Glut-1-Expression mit einem verlängerten metastasenfreien Überleben assoziiert. In ähnlicher Weise wurde auch für die CA IX-Expression eine positive Korrelation zur intratumoralen Hypoxie im Zervixkarzinom gezeigt (56) und ein Zusammenhang zwischen der CA IX-Expression und einer ungünstigen Prognose beim Zervix-, Mamma- und Lungenkarzinom nachgewiesen (57-59).

Demgegenüber konnten andere Autoren den Zusammenhang zwischen der Glut-1-Expression wie auch der CA IX-Expression zur intratumoralen Hypoxie in den von ihnen untersuchten Zervixkarzinomen nicht nachweisen (60-62). Auch der *in vitro* beobachtete Zusammenhang zwischen der VEGF-Expression und Hypoxie konnte in Zervixkarzinomen nicht nachvollzogen werden (63). Eine weitere Studie von Mayer et al. (64) an Zervixkarzinombiopsien mit definiertem pO_2 konnte ebenso keine Beziehung des Transkriptionsfaktors HIF-1 zum Grad der aktuellen intratumoralen Hypoxie zeigen. Eine Ursache hierfür stellt die Tatsache dar, dass HIF-1 auch hypoxie-unabhängig induziert werden kann, z.B. durch Aktivierung von Onkogenen, Verlust von Tumorsuppressorgenen und Zytokine (18).

Diese Diskrepanz zwischen den über kurze Zeiträume *in vitro* beobachteten Zusammenhängen und der in einem klinischen Tumor vorherrschenden Situation deutet auf eine komplexere Regulierung der hypoxie-induzierten Gene hin.

Die Hypoxie in soliden Tumoren resultiert aus einem gesteigerten Sauerstoffbedarf des wachsenden Malignoms, das bald seine Sauerstoffversorgung überschreitet, kombiniert mit verlängerten Diffusionswegen, einer gestörten Mikrogefäßfunktion und tumorbedingter Anämie (9). Diese Imbalance kann in hypoxischen Geweben durch eine Hochregulierung von angiogenen Faktoren, wie z.B. VEGF ausgeglichen werden (12). Das daraufhin entstehende Tumorgefäßnetz weist jedoch eine pathologische und chaotische Gefäßarchitektur auf, die letztlich das hypoxische Mikromilieu und damit auch die hypoxische Genaktivierung aufrechterhalten kann (20). Auf der anderen Seite kommt es durch den Wechsel von Thrombosen und Fibrinolysen in den neu aussprießenden Gefäßen zu

Perioden transienter Reperfusion mit der entsprechenden Reoxygenierung. Diese Komplexität solider Tumoren wird mit den üblicherweise verwendeten *In-vitro*-Modellen nur unzureichend abgebildet, da diese typischerweise aus einer einmaligen Hypoxie-Phase von bis zu 48 Stunden bestehen (65, 66).

In den folgenden Kapiteln werden eigene Untersuchungen zum Zusammenhang zwischen hypoxie-induzierten Markern und intratumoraler Oxygenierung in Zervixkarzinomen dargestellt.

Voraussetzung unserer Untersuchungen ist die korrekte Messung der intratumoralen Oxygenierung ($pO_{2;}$ (40)). Diese Untersuchungen wurden von der Ethikkommission der Medizinischen Fakultät der Universität Leipzig genehmigt. Patientinnen mit Zervixkarzinom, die sich an der Universitätsfrauenklinik vorstellen, wurden über die pO_2-Messung aufgeklärt und nach ihrer schriftlichen Einwilligung in die Studie eingeschlossen.

Als Goldstandard der intratumoralen pO_2-Messung wird die Messung mit Hilfe einer Eppendorf-Elektrode innerhalb des makroskopisch vitalen Tumorgewebes angesehen (40). Dabei wird mit der Sauerstoffsonde mindestens entlang zweier verschiedener Kanäle im Tumor gemessen. Die Sonde beginnt in ca. 5 mm Tiefe und sammelt pro Kanal ca. 30 Datenpunkte. Direkt im Anschluss an die Messung wird mittels Hochgeschwindigkeits-Stanzbiopsie das Gewebe des zuvor gemessenen Kanals gewonnen. Die Biopsien werden formalinfixiert und in Paraffin eingebettet und in Kooperation mit dem Institut für Pathologie kann somit für jede Messung bestätigt werden, ob im vitalen Tumorgewebe oder innerhalb von Nekrosen oder tumorfreien Arealen gemessen wurde.

Diese Herangehensweise hat noch einen weiteren entscheidenden Vorteil: Die anschließenden immunhistochemischen Untersuchungen können dann an Gewebe mit genau definiertem Oxygenierungsgrad durchgeführt werden, da nur die Messpunkte der zugehörigen Biopsie verwendet werden. Damit erlaubt diese Methode eine präzisere Beschreibung der räumlichen Beziehung zwischen der Expression bestimmter Genprodukte und der intratumoralen Oxygenierung als alternative Methoden, die die Proteinexpression in einer Gewebebiopsie zur Oxygenierung des gesamten Tumors in Beziehung setzen.

2.2.1 Tumorhypoxie und c-Met-Expression im Zervixkarzinom

Leo C, Horn LC, Einenkel J, Hentschel B, Höckel M.
Tumor hypoxia and expression of c-met in cervical cancer.
Gynecol Oncol. 2007 Jan;104(1):181-5.

Hypoxie trägt in verschiedener Hinsicht zur malignen Progression bei. Ein Aspekt ist offenbar die Erhöhung der Invasivität und Förderung der Metastasierung der betreffenden Tumoren. Dem Protoonkogen c-Met kommt eine Schlüsselfunktion bei der Kontrolle von Wachstumsvorgängen, Invasivität und Metastasierung von Krebszellen zu. c-Met ist eine membranständige Tyrosinkinase, die als Rezeptor für den Hepatocyte Growth Factor (HGF, scatter factor-1) fungiert. Eine fehlerhafte Aktivierung des HGF-Pathways wurde in verschiedenen Tumorentitäten für das invasive Wachtum und die Bildung von Metastasen mitverantwortlich gemacht. c-Met selbst wird in einer Vielzahl von soliden Tumoren überexprimiert, so z.B. im Brustkrebs, in Nasopharynxkarzinomen, Kolon- und Zervixkarzinomen (67-70). Die Arbeitsgruppe von Penacchietti et al. zeigte u.a. in der Zervixkarzinom-Zelllinie SiHa, dass Hypoxie *in vitro* die Transkription des c-Met-Protooncogens aktiviert, was letztlich in höheren Proteinmengen resultierte (23). In der vorliegenden Studie haben wir daher die Expression von c-Met in Zervixkarzinomen untersucht und die Beziehung zwischen c-Met und intratumoraler Hypoxie bzw. klinisch-pathologischen Parametern analysiert. Zervixkarzinombiopsien von 43 Patientinnen, bei denen mit der Eppendorf-Elektrode der intratumorale pO_2 gemessen worden war, standen für die immunhistochemischen Untersuchungen mit einem polyklonalen Antikörper gegen c-Met zur Verfügung. Eine c-Met-Expression fand sich in 72% der untersuchten Zervixkarzinome. In schlecht differenzierten G3-Tumoren fand sich eine signifikant stärkere c-Met-Expression ($p=0,03$). Ausserdem war die c-Met-Expression signifikant mit einem netzig-infiltrativen Invasionsmuster assoziiert ($p=0,008$), welches prognostisch ungünstiger ist. Allerdings beobachteten wir keinen Zusammenhang zwischen der c-Met-Expression und der intratumoralen Hypoxie, dem Vorhandensein von Lymphgefäßeinbrüchen und dem Gesamtüberleben. Unsere Ergebnisse deuten auf zusätzliche Mechanismen der c-Met-Aktivierung *in vivo* hin. Trotz seiner *in vitro* beobachteten Hypoxie-Induktion ist die Expression von c-Met in etablierten Tumoren offenbar vom aktuellen intratumoralen Sauerstoffpartialdruck unabhängig.

2.2.2 Tumorhypoxie und Expression des proapoptotischen BNIP3 im Zervixkarzinom

Leo C, Horn LC, Höckel M.
Hypoxia and expression of the proapoptotic regulator BNIP3 in cervical cancer.
Int J Gynecol Cancer. 2006; 16, 1–7.

In Studien an Tumorzelllinien war gezeigt worden, dass das Bcl-2/adenovirus E1B19kd-interacting protein 3 (BNIP3) unter hypoxischen Bedingungen *in vitro* hochreguliert wird (32, 33). Weiterhin weist BNIP3 in Brustkrebsgeweben im Vergleich zu normalem Brustgewebe eine starke Expression auf (33). BNIP3 ist ein mitochondriales Protein und proapoptotisches Mitglied der Bcl-2-Familie. In der hier zusammengefassten Arbeit werden die Expression von BNIP3 im Zervixkarzinom sowie seine Beziehung zur intratumoralen Oxygenierung und klinisch-pathologischen Parametern erstmals untersucht.

Dazu wurden zunächst die Zervixkarzinom-Zelllinien SiHa, Caski und C33a unter hypoxischen Bedingungen (0,1% O_2) kultiviert. Nach definierten Zeitpunkten (0h, 2h, 6h, 12h, 24h) wurde RNA isoliert und im Northern Blot analysiert. Die BNIP3 mRNA wurde unter Hypoxie in allen drei Zervixkarzinom-Zelllinien stark hochreguliert mit einem Maximum nach 12 Stunden. Zu diesem Zeitpunkt war ein 14 bis 17facher Anstieg der BNIP3-mRNA im Vergleich mit dem 0-Stunden-Zeitpunkt zu verzeichnen. Des Weiteren standen uns 50 Zervixkarzinombiopsien mit definiertem pO_2 für die immunhistochemische Untersuchung mit einem polyklonalen Antikörper gegen BNIP3 zur Verfügung. Die Biopsien stammten von Patientinnen mit Zervixkarzinom, bei denen im Rahmen der unter 2.2 beschriebenen Studie eine intratumorale Sauerstoffmessung mit der Eppendorf-Elektrode durchgeführt worden war. In 82% der Zervixkarzinome wurde BNIP3 exprimiert. Tumoren mit höheren FIGO-Stadien wiesen eine signifikant stärkere BNIP3-Expression auf (p=0,028). Wir fanden jedoch keine Korrelation zwischen der BNIP3-Expression und dem intratumoralen pO_2. Zusätzlich wurde von weiteren sieben Patientinnen zum Zeitpunkt der Operation sowohl Zervixkarzinomgewebe als auch Gewebe der tumorfreien Zervix gewonnen. Hier war eine im Vergleich zum gesunden Zervixgewebe erhöhte BNIP3-mRNA-Expression in fünf von sieben Karzinomen zu verzeichnen. Obwohl BNIP3 *in vitro* deutlich hypoxie-induzierbar ist, weisen unsere Ergebnisse auf zusätzliche Regulationsmechanismen *in vivo* hin. Unsere Studie unterstreicht wiederum die Diskrepanz zwischen *In-vitro*-Modellen der Tumorhypoxie und der Komplexität solider Tumoren.

2.2.3 Die Expression von Erythropoietin und Erythropoietin-Rezeptor im Zervixkarzinom und ihre Beziehung zu Überleben, Hypoxie und Apoptose

Leo C, Horn LC, Rauscher C, Hentschel B, Liebmann A, Hildebrandt G, Höckel M. Expression of erythropoietin and erythropoietin receptor in cervical cancer and relationship to survival, hypoxia and apoptosis.
Clin Cancer Res. 2006; 12:6894-6900.

Hypoxie stellt einen physiologischen Stimulus für die Expression von Erythropoietin (Epo) dar. Epo ist ein Glycoprotein-Hormon, das die Erythropoiese stimuliert. Es wird in der Niere und zu einem kleineren Teil auch in der Leber gebildet und wirkt über den Erythropoietin-Rezeptor (EpoR) an hämatopoietischen Vorläuferzellen, wo es Wachstum stimuliert, Apoptose verhindert und die Differenzierung zu Erythrozyten induziert. Die Hypoxie-Induzierbarkeit von Epo wird über den Transkriptionsfaktor HIF-1 vermittelt. Auch EpoR wird durch Hypoxie hochreguliert, allerdings über HIF-1-unabhängige Mechanismen (71). Neuere Studien haben Epo und EpoR in verschiedenen soliden Tumoren nachgewiesen, z.B. im Zervixkarzinom, Endometriumkarzinom, Brustkrebs, in Kopf-Hals-Tumoren und Lungenkarzinomen (71-77).

Um die Rolle des Epo/EpoR-Systems für die Biologie und Klinik solider Tumoren zu analysieren, haben wir in der in diesem Kapitel beschriebenen Arbeit die Expression von Epo und EpoR im Zervixkarzinom und ihre Beziehung zur intratumoralen Hypoxie, Proliferation, Apoptose sowie klinisch-pathologischen Parametern einschließlich dem Überleben untersucht.

Dazu stand uns Zervixkarzinomgewebe von 48 Patientinnen mit intratumoraler pO_2-Messung zur Verfügung, welches wir mit Antikörpern gegen Epo, EpoR und Ki-67 sowie mit TUNEL-Assays analysierten. Epo wurde in 88% und EpoR in 92% der untersuchten Karzinome nachgewiesen. Zervixkarzinome mit starker Epo-Expression waren mit einem signifikant schlechteren Gesamtüberleben assoziiert (3-Jahresüberlebensrate: 50,0% vs. 80,6%; p=0,0084). Der Epo-Effekt war unabhängig vom FIGO-Stadium und der Therapieart, wie wir in der multivariaten Cox-Regressionsanalyse zeigen konnten. Auch das rezidivfreie Überleben war bei Patientinnen mit stark Epo-exprimierenden Zervixkarzinomen signifikant kürzer (3- Jahresüberlebensrate: 53,6% vs. 80,8%; p=0,043).

Die Stärke der EpoR-Expression korrelierte signifikant mit der Tumorgröße und war weiterhin signifikant mit dem Vorhandensein von Lymphbahneinbrüchen assoziiert. Eine Korrelation zwischen Epo- bzw. EpoR-Expression und der intraumoralen Hypoxie beobachteten wir hingegen nicht.

Des Weiteren zeigte sich sowohl zwischen der Epo- als auch EpoR-Expression und der Apoptoserate ein positiver signifikanter Zusammenhang (Epo: r=0,49, p=0,001; EpoR: r=0,36, p=0,021). Möglicherweise dient die Hochregulation des Epo/EpoR-Systems als

Kompensationsmechanismus für die hohen Apoptoseraten, die in einigen der von uns untersuchten Zervixkarzinome beobachtet wurden, um dann letztlich anti-apoptotische Effekte zu vermitteln. Diese Hypothese ist konsistent mit der von uns beobachteten schlechteren Prognose von Patientinnen mit stark Epo-exprimierenden Zervixkarzinomen. Weiterhin könnte mit dieser Hypothese der negative Effekt von rekombinantem humanen Epo (rhuEpo) auf die Prognose von Patienten mit Kopf-Hals-Tumoren und Mammakarzinomen erklärt werden, der in zwei großen klinischen Studien postuliert wurde (78, 79), da exogenes Epo in einem schon stimulierten Epo/EpoR-System das Tumorwachstum weiter verstärken könnte.

In der hier beschriebenen Arbeit haben wir erstmals den Zusammenhang zwischen der Epo- bzw. EpoR-Expression im Zervixkarzinom und der Prognose, der intratumoralen Hypoxie sowie der Apoptose untersucht. Unsere Ergebnisse deuten auf eine kritische Rolle des endogenen Epo/EpoR-Systems im Zervixkarzinom hin, die aber offenbar von einer aktuell vorherrschenden Hypoxie unabhängig ist.

2.3 Einführung in die Arbeiten 2.3.1 – 2.3.2

Unter physiologischen Umständen stellt Hypoxie einen Stimulus zur Apoptose dar. Jedoch scheint es eine Subgruppe von Zervixkarzinomen zu geben, die in der Lage sind, der hypoxie-induzierten Apoptose zu entgehen. So konnten Höckel et al. zeigen, dass es eine Gruppe von hypoxischen Zervixkarzinomen gibt, die trotz ausgeprägter intratumoraler Hypoxie nur eine geringe Apoptoserate aufweisen (37). Diese Tumoren waren im Vergleich mit den hypoxischen Zervixkarzinomen mit hoher Apoptoserate bzw. den nicht-hypoxischen Zervixkarzinomen mit einem aggressiveren Phänotyp assoziiert, der in einer schlechteren Prognose resultierte.

Befunde aus *In-vitro*-Untersuchungen und Tiermodellen legen nahe, dass die Tumorhypoxie die maligne Progression über die Initiierung von Selektionsprozessen beeinflusst. Ein wichtiger Mechanismus hierbei ist vermutlich die klonale Selektion von Tumorzellen, die ihre Fähigkeit zur Apoptose verloren haben. So untersuchten Kim et al. humane Zervixepithelzellen, die die HPV-16-Onkoproteine E6 und E7 exprimierten (80). Wurden diese Zellen Hypoxie ausgesetzt, kam es zur Selektion von Subpopulationen, die ein vermindertes Apoptosepotential besaßen. Weiterhin zeigten Graeber et al. in einem Mausmodell, dass hypoxische Bedingungen zu einer Selektion von apoptose-resistenten p53-negativen MEFs (Mausembryo-Fibroblasten) führten (38).

Die hypoxie-vermittelte Apoptose wird über den mitochondrialen Apoptose-Pathway ausgeführt. Eine zentrale Stellung innerhalb dieses Pfades nimmt der Apoptotic-Protease-Activating-Factor-1 (Apaf-1) ein. Apaf-1 ist essentieller Bestandteil des Apoptosoms, welches als Antwort auf diverse zelluläre Stressfaktoren (wie z.B. Hypoxie, DNA damage und Onkogenaktivierung) gebildet wird. Die genannten Stressfaktoren führen über den intrinsischen mitochondrialen Apoptose-Pathway zur Aktivierung von Caspasen, was letztlich im programmierten Zelltod mündet (81-83). Apaf-1-Knockout-MEFs wiesen schwerwiegende Defekte in ihrer apoptotischen Reaktion gegenüber hypoxischer Stimulierung auf (39). Damit wurde gezeigt, dass Apaf-1 *in vitro* eine essentielle Komponente für die hypoxie-vermittelte Apoptose darstellt.

Die Mechanismen, die einer hypoxie-induzierten Apoptoseresistenz in klinischen Tumoren zugrunde liegen, sind bisher unbekannt. Interessanterweise wurde in den letzten Jahren aber in verschiedenen Malignomen eine Hypermethylierung des Apaf-1-Promotors nachgewiesen, die in einem Verlust der Apaf-1-Expression resultierte (84-86).

Im nächsten Kapitel werden zwei Arbeiten zusammengefasst, die sich mit der Expression von Apaf-1 im Zervixkarzinom befassen und die Rolle von Apaf-1 bei der hypoxie-induzierten Apoptoseresistenz untersuchen.

2.3.1.- 2.3.2 Der Apoptosevermittler Apaf-1 und seine Rolle bei der Hypoxie-induzierten Apoptoseresistenz im Zervixkarzinom

Leo C, Richter C, Horn LC, Schütz A, Pilch H, Höckel M.
Expression of Apaf-1 in cervical cancer correlates with lymph node metastasis but not with intratumoral hypoxia.
Gynecol Oncol. 2005; 97(2):602-6.

Leo C, Horn LC, Rauscher C, Hentschel B, Richter CE, Schütz A, Leo CP, Höckel M.
Lack of Apaf-1 expression and resistance to hypoxia-induced apoptosis in cervical cancer. Clin Cancer Res. 2007;13(4):1149-53.

In den hier zusammengefassten Arbeiten wurden erstmals die Expression von Apaf-1 im Zervixkarzinom und seine Beziehung zu klinisch-pathologischen Parametern, zur Hypoxie und zur Apoptose untersucht.

Dazu stand uns Gewebe von 86 Zervixkarzinomen zur Verfügung, bei denen zuvor mittels Eppendorf-Elektrode der intratumorale pO_2 gemessen worden war. Die immunhistochemischen Untersuchungen erfolgten mit einem polyklonalen Antikörper gegen Apaf-1. Wir demonstrierten, dass Apaf-1 im Großteil der Zervixkarzinome exprimiert wird. Zervixkarzinome mit schwacher oder fehlender Apaf-1-Expression wiesen zum Zeitpunkt der Tumorchirurgie signifikant häufiger Lymphknotenmetastasen auf (p=0,022). Zwischen der Apaf-1-Expression und dem FIGO-Stadium, pT-Stadium, Lymphbahneinbrüchen und histologischen Grading fand sich kein Zusammenhang. Auch fand sich keine direkte Korrelation zur intratumoralen Hypoxie. Von 56 der Patientinnen standen uns Zervixkarzinombiopsien mit definiertem pO_2 für eine Analyse der Apoptoserate mittels TUNEL-Assays zur Verfügung. In diesen Karzinomen untersuchten wir den Zusammenhang zwischen der Apaf-1-Expression, der Hypoxie und der Apoptoserate. Wir beschreiben eine Subgruppe von 16 Zervixkarzinomen, die trotz ausgeprägter intratumoraler Hypoxie eine niedrige Apoptoserate aufwiesen. Dieser Subgruppe gehörten sechs der insgesamt acht Apaf-1-negativen Zervixkarzinome an. Damit waren 37.5% der Karzinome in dieser hypoxischen niedrig-apoptotischen Gruppe Apaf-1-negativ im Vergleich mit nur 5.0% in der Gruppe der hypoxischen stark-apoptotischen und normoxischen Zervixkarzinome. (p=0.005).

Bei einer Nachbeobachtungszeit von 44 Monaten zeigte sich ein Trend zu einer schlechteren Prognose in der Gruppe der hypoxischen niedrig-apoptotischen Zervixkarzinome, der jedoch keine statistische Signifikanz erreichte (p=0.08).

Unsere Daten zeigen einen Apaf-1-Verlust in einem signifikanten Anteil von Tumoren, die trotz Hypoxie eine niedrige Apoptoserate aufweisen. Aufgrund dieses Befundes schlagen wir einen Mechanismus vor, über den hypoxische Zervixkarzinome eine Apoptoseresistenz erlangen könnten. Zudem deutet die häufigere Lymphknotenmetastasierung in Apaf-1-

negativen Karzinomen darauf hin, dass der Apaf-1-Verlust möglicherweise einen Marker für ein aggressiveres Tumorverhalten darstellt.

3. Diskussion und Ausblick

Klinische Beobachtungen an verschiedenen Tumorentitäten zeigten, dass die intratumorale Hypoxie mit klinisch aggressiveren Phänotypen assoziiert ist, die einer therapeutischen Beeinflussung schwerer zugänglich sind und bei denen eine ungünstige Prognose der betroffenen Patienten nachgewiesen werden konnte (5-7). Höckel et al. konnten die Hypoxie als unabhängigen Indikator für eine schlechtere Prognose bei Patientinnen mit Zervixkarzinomen identifizieren (5). Die hier zusammengefassten Arbeiten beschäftigen sich mit den molekularen Mechanismen dieser - vermutlich hypoxie-vermittelten - malignen Progression.

In den ersten vier Arbeiten konnte gezeigt werden, dass Tumorzellen *in vitro* auf kurzfristige Hypoxie mit einer Anpassung der Genexpression reagieren, die *in vivo* einen Überlebensvorteil und erhöhte Aggressivität erwarten ließe. Diese Adaptation der Genexpression wird hauptsächlich über den hypoxie-induzierbaren Transkriptionsfaktor HIF-1 vermittelt (87) und ist reversibel. Wie auch andere Arbeitsgruppen gezeigt haben, induziert HIF-1 *in vitro* die Expression einer Vielzahl von Genen, die aufgrund der Funktion ihrer Proteinprodukte im Wesentlichen vier verschiedenen funktionellen Gruppen zugeordnet werden können: dem Metabolismus, der Angiogenese, der Invasion/Metastasierung und der Apoptose (23, 27, 32, 88, 89). Inwieweit diese Veränderungen tatsächlich dazu beitragen, dass klinische Tumoren einen „hypoxischen Phänotyp“ annehmen, indem sie aggressiver werden und eine Resistenz gegenüber verschiedenen Therapiemodalitäten erwerben können, lässt sich jedoch anhand von *In-vitro*-Untersuchungen nicht beantworten.

Daher haben wir in den hier besprochenen Arbeiten (2.2.1 -2.2.3) die Expression von c-Met, BNIP3, Epo und EpoR im Zervixkarzinom untersucht, für die eine deutliche Hypoxie-Induzierbarkeit *in vitro* bekannt ist und die – auf jeweils verschiedene Weise - zum Prozess der malignen Progression beitragen könnten. Dabei war eine Zielstellung, den Zusammenhang zwischen der Expression dieser Marker *in vivo* und der intratumoralen Oxygenierung zu untersuchen. Wir konnten zeigen, dass klinische Tumoren eine vom aktuellen intratumoralen Oxygenierungsgrad unabhängige Expression dieser hypoxie-induzierbaren Gene/Proteine aufweisen, die mit der Aggressivität der Tumoren korrelierte. Diese Beobachtung deutet auf zusätzliche Regulationsmechanismen der entsprechenden Gene bzw. Proteine *in vivo* hin und demonstriert eine Diskrepanz zwischen *In-vitro*-Modellen der Tumorhypoxie und der biologischen Komplexität solider Tumoren. Diese Komplexität wird mit den üblicherweise verwendeten *In-vitro*-Modellen nur unzureichend abgebildet, da diese typischerweise aus einer einmaligen Hypoxie-Phase von bis zu 48 Stunden bestehen (65, 66) und damit den Einfluss chronischer Hypoxie und den Effekt von Phasen der Reoxygenierung völlig außer acht lassen. Ein weiterer Grund für die fehlende Hypoxieabhängigkeit *in vivo* kann auch darin liegen, dass Epo, c-Met und BNIP3 durch den

Transkriptionsfaktor HIF-1 induziert werden. Dieser wird zwar sehr robust und schnell im hypoxischen Milieu hochreguliert, kann aber auch hypoxie-unabhängig induziert werden, z.B. durch Aktivierung von Onkogenen, Verlust von Tumorsuppressorgenen und durch Zytokine (11, 18). Zwar konnten wir keinen Zusammenhang zwischen der Expression der genannten Proteine und der Tumorhypoxie *in vivo* nachweisen, jedoch fanden sich für alle untersuchten Marker signifikante Zusammenhänge mit verschiedenen klinisch-pathologischen Parametern, die ihrerseits wiederum mit einem aggressiveren Tumorverhalten assoziiert sind: Die c-Met-Expression war signifikant mit einer schlechteren Differenzierung und einem netzig-infiltrativen Invasionsmuster assoziiert, also mit Tumorcharakteristika, die einen aggressiveren Phänotyp repräsentieren. Die BNIP3-Expression war in Zervixkarzinomen höherer FIGO-Stadien signifikant stärker. Ob dies ein Effekt der fortgeschrittenen Tumorprogression ist oder ob sich BNIP3 als prognostischer Marker im Zervixkarzinom eignet, muss in weiteren Studien geklärt werden. Die Arbeitsgruppe von Giatromanolaki (90) hat das proapoptotische BNIP3 als prognostischen Marker für ein schlechteres Überleben bei Patienten mit nicht-kleinzelligem Lungenkarzinom identifiziert. Auch für EpoR konnten wir in den von uns untersuchten Zervixkarzinomen signifikante Korrelationen mit klinisch-pathologischen Markern aggressiven Tumorverhaltens, wie Tumorgröße und Lymphgefäßeinbrüchen nachweisen. Ausserdem waren Zervixkarzinome mit starker Epo-Expression mit einem signifikant schlechteren Gesamtüberleben und rezidivfreien Überleben assoziiert. Diese Beobachtungen legen den Schluss nahe, dass im Verlauf der Tumorigenese und Tumorprogression verschiedene hypoxie- und reoxygenierungs-abhängige Veränderungen in Tumorzellen stattfinden. Diese resultieren letztlich in längerfristigen, vermutlich irreversiblen Änderungen der Expression hypoxie-induzierter Gene, die auch nach Wegfall des hypoxischen Stimulus bestehen bleiben und damit einen „fixierten hypoxischen Phänotyp“ zur Folge haben.

Ein weiteres Ziel unserer Arbeiten war es, die klinisch beobachtete Apoptoseresistenz in hypoxischen Zervixkarzinomen und die ihr zugrunde liegenden Mechanismen näher zu untersuchen. Bisher war über die molekularen Ursachen dieser Apoptoseresistenz wenig bekannt. Die hypoxie-vermittelte Apoptose wird über den mitochondrialen Apoptose-Pathway ausgeführt. Apaf-1 nimmt in diesem Pathway eine zentrale Stellung ein. Zellen von Apaf-1-Knockout-Mäusen wiesen schwerwiegende Defekte in ihrer apoptotischen Reaktion gegenüber hypoxischer Stimulierung auf (39), womit gezeigt wurde, dass Apaf-1 *in vitro* eine essentielle Komponente für die hypoxie-vermittelte Apoptose darstellt. In den hier vorgelegten Arbeiten wurden erstmals die Expression von Apaf-1 im Zervixkarzinom und ihre Beziehung zu Hypoxie, klinisch-pathologischen Parametern und Apoptose untersucht. Die gewonnenen Daten legen nahe, dass der Verlust der Apaf-1-Expression einen Mechanismus für die beobachtete Apoptoseresistenz in hypoxischen Zervixkarzinomen darstellen könnte.

Damit leisten diese Arbeiten einen wesentlichen Beitrag zur Aufklärung der molekularen Ursachen der hypoxie-induzierten Apoptoseresistenz und damit der schlechteren klinischen Prognose hypoxischer Tumoren. Auch das signifikant häufigere Auftreten von Lymphknotenmetastasen legt nahe, dass der Apaf-1-Verlust ein Marker für aggressives Tumorverhalten ist und dass eine niedrige Apaf-1-Expression möglicherweise einen ungünstigen prognostischen Faktor darstellt.

Für verschiedene Tumorarten konnte in den letzten Jahren ein Apaf-1-Verlust gezeigt werden, so für das maligne Melanom, das Blasenkarzinom und eine Form der akuten lymphoblastischen Leukämie (84-86). Die Ursache für den Apaf-1-Verlust lag dabei in einer Hypermethylierung des Apaf-1-Promoters, ein möglicher Zusammenhang mit der Tumorhypoxie wurde bei diesen Tumorentitäten jedoch bisher nicht untersucht. Ob diese Form des epigenetischen Silencing auch für den Verlust der Apaf-1-Expression bei den hypoxischen Zervixkarzinomen verantwortlich ist, muss in weiteren Studien untersucht werden.

Insgesamt lässt sich aus den hier zusammengefassten Arbeiten – in Zusammenschau mit den Vorarbeiten aus unserer und anderen Arbeitsgruppen - folgende Hypothese des Tumorverhaltens unter Hypoxie (und intermittierender Reoxygenierung) ableiten.

(1) Zunächst kommt es zu Anpassungen auf dem Proteom-Level, die durch Adaptation der Genexpression sowie durch posttranskriptionale und posttranslationale Modifikationen hervorgerufen werden und die nach dem Wegfall der Hypoxie reversibel sind. (2) Auf dem Genom-/Epigenom-Level kommt es zu einer hypoxie- und reoxygenierungs-bedingten Steigerung der genomischen und epigenetischen Instabilität, was zu einer Generierung von Tumorzellvarianten führt. (3) Unter dem Selektionsdruck des herrschenden Mikromilieus kommt es dann im klinischen Tumor zu einer Selektion und klonalen Expansion von Zellpopulationen, in denen der hypoxische (=aggressive) Phänotyp irreversibel genetisch fixiert und damit auch unabhängig vom Vorliegen einer tatsächlichen intratumoralen Hypoxie ist. Parallel dazu findet unter Hypoxie eine Auslese von apoptose-resistenten Tumorzellklonen statt, die in der Folge nicht nur gegenüber der Hypoxie, sondern auch gegenüber anderen Apoptose-Reizen (Bestrahlung, Chemotherapie) weniger empfindlich ist (91)(Abb. 2).

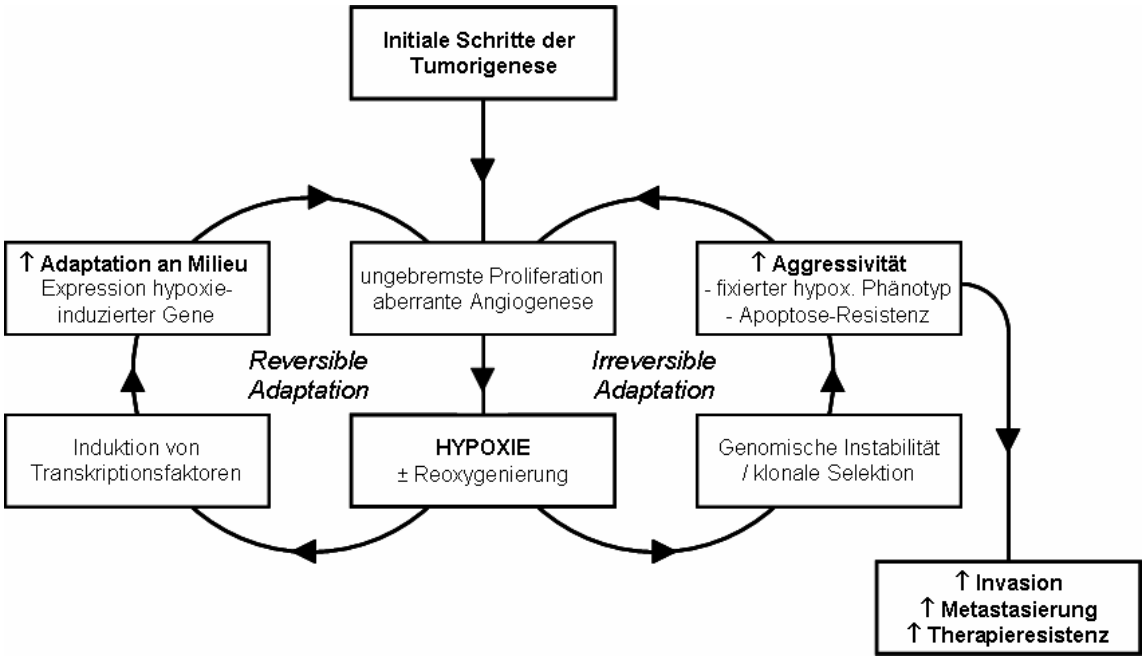

Abbildung 2: Hypothese der Hypoxie-Reoxygenierungs-getriebenen Tumorprogression

Aufgrund ihres häufigen Vorkommens in soliden Neoplasien, kann die Tumorhypoxie möglicherweise gezielt als Ansatzpunkt für die Tumortherapie genutzt werden. Vier Strategien sind momentan in frühen klinischen und präklinischen Studien: die Aktivierung zytostatischer Prodrugs durch Hypoxie, die hypoxie-spezifische Gentherapie, das Targeting des Transkriptionsfaktors HIF-1 und der Einsatz rekombinanter obligat anaerober Bakterien (92). Mit Hilfe dieser viel versprechenden Ansätze könnte es gelingen, die prognostisch ungünstige Tumorhypoxie als Basis für die Entwicklung neuer onkologischer Therapien zu nutzen.

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The Hypoxic Tumor Microenvironment and Gene Expression

Cornelia Leo, Amato J. Giaccia, and Nicholas C. Denko

Solid tumors are not static entities but are constantly responding to environmental signals as they grow and develop. One mechanism by which they respond to the adverse conditions of the tumor microenvironment is through coordinated changes in gene expression. The synchronized turning of genes on and off leads to biologic adaption to the adverse oxygen-poor environment. Because tumor hypoxia can be found in almost every solid tumor, it represents one of the most pervasive microenvironmental stresses that can impact malignant progression and therapeutic response. Interestingly, tumors that exhibit robust induction of hypoxia-responsive gene expression networks show a clinically more aggressive natural history. The contribution of hypoxia-responsive gene networks to malignant response is currently under investigation. An understanding of the coordinated functions of hypoxia induced and repressed genes can lead to a better understanding of the clinical significance of the hypoxic tumor phenotype.

Hypoxia plays an important role in a variety of physiological as well as pathophysiological processes. Its biological relevance in the malignant progression of tumors has been a focus of investigation for more than a decade. Over the last several years, clinical studies have shown that hypoxia is an independent prognostic indicator of poor patient survival in different tumor types, including cervical carcinoma, head and neck cancer, and soft-tissue sarcomas.[1-4] Because this observation also holds true for surgically treated patients, it suggests that there are fundamental biological differences between hypoxic and nonhypoxic tumor cells.[1] A major mechanism by which hypoxia confers its effects is by differential regulation of gene expression. The most robust hypoxia-induced transcription factor is hypoxia-inducible factor 1 (HIF-1).[5] Genes that are induced by hypoxia can be grouped based on the function of the protein products, and these coordinately regulated genes may render tumor cells more aggressive and/or resistant toward different treatment modalities. The changes in gene expression in the hypoxic tumors are thought to be the same changes that help normal cells to adapt to a hypoxic microenvironment under noncancerous conditions such as wound healing.[6] In addition to epigenetic changes in gene expression, hypoxia can initiate selection processes for especially hardy cells and by this means select for cells with genetically fixed features of the "hypoxic phenotype" that contribute to tumor progression.[7] In this review, selected hypoxia-induced genes are highlighted, and their relevance in tumor physiology is discussed.

Hypoxia Generated Under Physiological Circumstances

The capability to respond to low-oxygen conditions developed in an evolutionary manner and is necessary to compensate for decreased oxygen levels that may occur under physiological circumstances. For example, Chen and coworkers[8] showed that hypoxia was necessary for proper embryonic development in the rat. Hyperoxia led to developmental abnormalities and to altered cell death patterns. Likewise, the HIF-1α knockout mouse is severely compromised in its transcriptional response to hypoxia and is not viable, dying at embryonic day 9.5.[9,10] The generation of tissue hypoxia occurs when the supply of oxygen delivered from the blood vessels cannot meet the demand within the tissue. The clearest example of decreased oxygen supply is found during vascular damage when the blood supply is acutely cut off (such as wounding, stroke, or myocardial infarction). Conversely, hypoxia can arise from increased oxygen demand during prolonged exercise. If the blood supply is completely removed,

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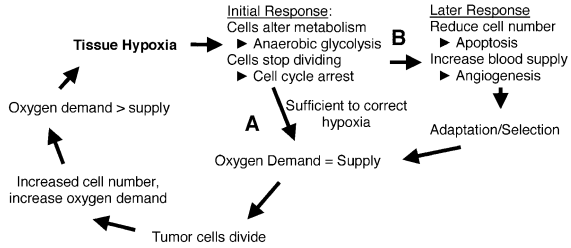

Figure 1. Pathophysiologic balance between oxygen supply and demand in a solid tumor. Tissue hypoxia occurs when supply cannot meet demand and elicits (A) early responses; if they are sufficient to establish normoxia, the cycle proceeds. If early responses are insufficient and hypoxia remains, then (B) late responses are initiated.

the tissue will eventually deplete all nutrients, with the oxygen being the first to be consumed.

The tissue responds to an oxygen deficit by both reducing the rate of oxygen consumed (decreased demand) and reestablishing a vascular supply (increased supply). Reduced demand can be achieved by decreasing cell number (hypoxia-induced cell cycle arrest, hypoxia-induced apoptosis), whereas increased supply is achieved by the generation of new blood vessels (hypoxic secretion of angiogenic compounds such as vascular endothelial growth factor [VEGF]). An additional driving force for the hypoxic cell is to maintain intracellular energy production at a level that is consistent with life. Mitochondrial function is significantly reduced under hypoxia, and the utilization of anaerobic glycolysis is highlighted by the large number of hypoxia-responsive genes that are involved in this function. In contrast to normal tissues in which these responses result in reestablishing a normoxic state, the hypoxic tumor never has sufficient vascularization to satisfy its oxygen demand and so the tumor exists in a chronic hypoxic state[11] (Fig 1). Determining the normal function(s) of hypoxia-responsive genes can therefore shed light on how the chronic hypoxic state within the tumor is contributing to the aggressive tumor behavior (Fig 2).

Hypoxia-Responsive Transcription Factors/HIF-1

The transcription factor with the most sensitive and specific induction in hypoxic conditions is HIF-1.[12] Over the past decade, this transcription factor has been the focus of intensive investigation. New insights into the function of HIF-1, the dissection of molecular mechanisms underlying oxygen sensing, and the identification of HIF-1 target genes contribute to a new understanding of the complex cellular response to hypoxia. Be-

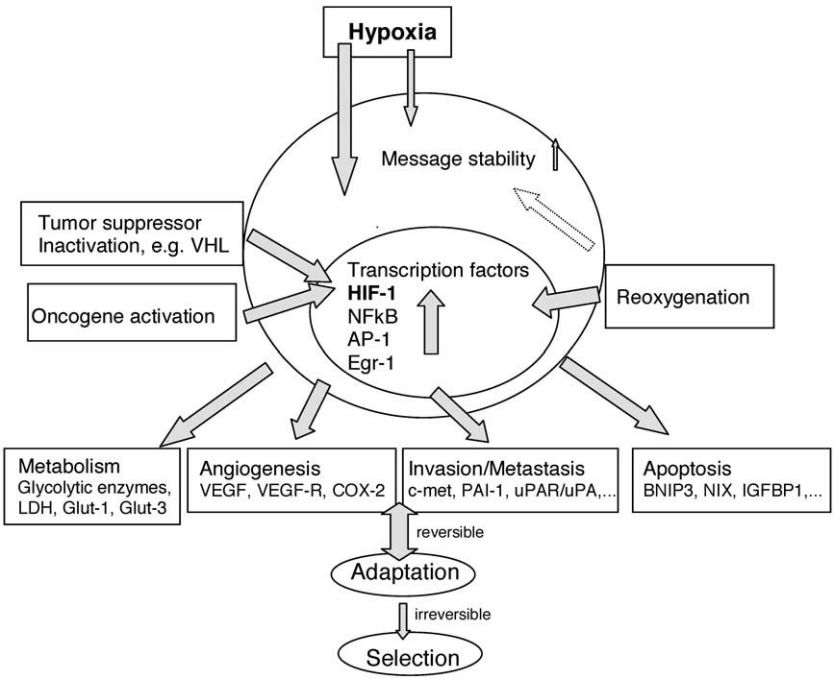

Figure 2. Molecular signals and responses in the hypoxic tumor cell. Hypoxia-responsive genes are grouped into putative functional categories.

cause areas of hypoxia can be found in nearly every solid tumor, these findings may lead to novel antitumor approaches.[13]

HIF-1 is a heterodimeric basic helix-loop-helix-PAS (PER/ARNT/SIM) transcription factor consisting of HIF-1α subunit and the constitutively expressed HIF-1β subunit. HIF-1 was first identified by analyzing the mechanism that leads to the induction of the erythropoietin gene.[14] Under hypoxic conditions, the normally labile HIF-1α protein becomes stabilized, leading to its rapid accumulation. On translocation to the nucleus, it heterodimerizes with the HIF-1β subunit, binds to specific DNA sequences within so-called hypoxia-response elements, and starts to transactivate specific target genes. By contrast, under normoxia, HIF-1α is rapidly targeted for degradation by the von Hippel-Lindau protein (VHL).[15] VHL only recognizes and binds HIF-1α after the latter is enzymatically hydroxylated on conserved prolyl residues within a domain termed the oxygen-degradation domain.[16,17] This process is carried out by a recently discovered family of prolyl hydroxylases that are oxygen dependent and require the cofactors 2- oxoglutarate, vitamin C, and iron.[18,19]

Additional downregulation of HIF-1 activity in the presence of oxygen is caused by the hydroxylation of the C-terminal transactivation domain, on a conserved asparagine residue 803. The responsible enzyme is the factor inhibiting HIF-1, and this hydroxylation prevents the recruitment of coactivators such as p300 and CBP.[20] In addition to the relatively well-characterized mechanisms of hypoxic HIF-1α subunit stabilization, oncogene activation, diverse growth factors, and cytokines are known to stabilize HIF-1α under normoxia via common cellular kinase pathways.[21]

Other transcription factors can be induced by hypoxia, such as NF-κB, AP-1, and early growth response factor 1. However, these are more general stress-responsive transcription factors whose response to hypoxia is less specific than that of HIF-1. In particular, NF-κB has been implicated as a mediator of the effects of hypoxia and reoxygenation in tumor cells.[22] It transactivates an extensive number of genes, among them cytokines and growth factors, acute-phase response proteins, leucocyte adhesion molecules, transcription/growth control factors, and immunoregulatory molecules.[23] The transcription factor early growth response factor 1 has also been shown to be induced in hypoxia, leading to an upregulation of tissue factor, thereby starting the procoagulant cascade.[24]

Additional Mechanisms of Hypoxic Gene Regulation

Besides the hypoxia-induced transcriptional activity through hypoxia-inducible transcription factors, posttranscriptional mechanisms leading to messenger RNA (mRNA) stabilization and translational control have been reported. Under hypoxic conditions, several hypoxia-induced mRNAs have been shown to become stabilized. This increased stability contributes to mRNA accumulation and is thought to be caused by mRNA-binding proteins interacting with elements in the 3′-untranslated regions.[25] The stabilizing element can be identified and transferred from erythropoietin, VEGF, and tyrosine hydroxylase mRNAs. Several proteins, polyCBP,[26] HuR,[27] and hnRNP L[28] have been implicated in this posttranscriptional mRNA induction.

Recent findings show that hypoxia can also actively repress gene expression, through both transcriptional and posttranscriptional mechanisms. The general transcriptional repressor negative cofactor 2 was recently shown to be activated in extracts from hypoxia-treated cells.[29] Reports of gene-specific hypoxic repression has also been identified that is mediated by the activity of either p53[30] or Dec1.[31] Furthermore, hypoxia can also repress mRNA translation to protein(s). Severe hypoxia seems to reduce translation through an endoplasmic reticulum stress signal of the PERK kinase.[32] These additional mechanisms suggest just examining the hypoxia-induced genes may yield an incomplete picture of gene expression changes in hypoxia.

Hypoxia-Regulated Gene Expression

Based on an extrapolation from expression profiling data, it has been estimated that approximately 1.5% of the genome is transcriptionally responsive to hypoxia.[33] Hypoxia-regulated genes are involved in diverse biological processes, some of which can be grouped into functional categories such as regulating metabolism, apoptosis, angiogenesis, or invasion. As a result, cancer cells undergo adaptive changes that allow them not

only to survive but even to proliferate or leave the adverse tumor micromilieu.

Metabolism

To adapt to hypoxia, cells switch from the (aerobic) citric acid cycle to anaerobic glycolysis to generate energy. Because of the reduced energy yield (of 2 adenosine triphosphate molecules from one glucose molecule by glycolysis in comparison to 38 adenosine triphosphate molecules from the citric acid cycle) the overall glucose consumption must increase. HIF-1α activates many enzymes of the glycolytic pathway,[34] as well as transporters responsible for accumulating glucose within the cell.[35] Another metabolic characteristic of cancer cells is their capability to survive and grow in low pH environments. The anaerobic consumption of glucose leads to the accumulation of lactic acid that results in intracellular and extracellular acidosis. Furthermore, the HIF-1–regulated induction of carbonic anhydrases IX and XII can also contribute to the regulation of the low extracellular pH environment of the tumor.[36]

Apoptosis

The effects of hypoxia on apoptosis are complex. Moderate levels of hypoxia (2% oxygen) are not generally apoptogenic, although they can induce HIF-responsive gene expression. However, severe hypoxia acts as a potent proapoptotic stimulus. Furthermore, prolonged hypoxia may even render tumors resistant to apoptosis.[7] It therefore seems probable that hypoxia can regulate the expression of both pro- and antiapoptotic molecules in a cell, and these combine with additional signals to result in the life/death decision. Because hypoxia can be an early event in tumor development, it can act as an early selection pressure on cancer cells to become resistant to hypoxia-induced apoptosis.[37] This acquired resistance to apoptosis can in turn contribute to the aggressive phenotype that is characteristic for many hypoxic tumors.

Hypoxia upregulates putative proapoptotic genes BNIP3 and NIX, in a p53-independent manner.[38] Although expression of these mitochondrial proteins can lead to apoptosis under normoxia, there is a discrepancy between their robust hypoxic induction in primary cells and a lack of apoptosis.[39] In cultured cardiomyocytes, it was shown that hypoxia alone was not sufficient to induce apoptosis, whereas the combination of hypoxia with acidosis caused apoptotic cell death.[40] It is also possible that overexpression of BNIP3/NIX is apoptotogenic only under normoxic conditions but has a different, still undefined role, under hypoxia.

The mechanisms by which hypoxia confers this apoptosis resistance have only partially been elucidated. In mouse models of minimally transformed fibroblasts rapid, p53-dependent apoptosis is observed that is signaled through the mitochondrial cytochrome C and Apaf.[41] The extreme sensitivity of these cells offers the possibility of selection for resistant variants.[7] These experimental data were supported by a study in cervical cancer patients by Hockel et al,[37] which showed that hypoxic tumors with a low apoptotic index are highly aggressive. Apoptotic signaling in this hypoxia-induced p53-dependent pathway requires Apaf-1 and Caspase-9 because cells from the knockout mice showed severe defects in the apoptotic response to hypoxia.[42]

Cells have also developed mechanisms to counteract hypoxia-induced cell death signals. One such mechanism is the hypoxic upregulation of the antiapoptotic gene IAP2 that resulted in death resistance of immortalized rat kidney proximal tubular epithelium.[43] Likewise, antiapoptotic bcl-w has also been seen elevated in hypoxia.[33]

Angiogenesis and Oxygen Delivery

In cancer, hypoxia results from the inexorable oxygen demand of the growing tumor, which sooner or later exceeds its blood supply. To respond to this condition, hypoxia coordinately induces several proangiogenic growth factors and represses antiangiogenic factors. The key to the synthesis of new vessels is the coordinated expression of the numerous angiogenic factors, most notably the HIF-1 target gene VEGF. Other genes involved in the growth of new vessels in a hypoxic environment include VEGF receptor 1 (Flt-1); PAI-1; angiopoietin-2; Tie-2; cyclooxygenase (COX)-1; COX-2; iNOS; adrenomedullin; FGF-3; monocyte chemotactic protein-1; osteopontin; histone deacetylase; TGFα, β1, and β3; and hepatocyte growth factor.[5] The large number of proangiogenic genes suggests that a coordinated pattern of expression is necessary for a functional vessel. In addition, hypoxia inhibits antiangiogenic factors such as thrombospondin I and II.[33] The tumor vasculature, on the other

hand, is characterized by a pathologic and chaotic architecture leading to an impaired function that maintains the hypoxic micromilieu and thus hypoxic gene activation. The sprouting of pathological vessels and thrombosis, embolism, and fibrinolysis has been thought to lead to areas of transient reoxygenation

The gene for COX-2 was shown to be induced by hypoxia via the transcription factor NF-κB.[44] COX-2 is an interesting molecule because it supports angiogenesis by upregulating VEGF[45] and inhibits apoptosis by inducing Bcl-2.[46] Overexpression of COX-2 was shown in a variety of tumor entities and is generally associated with poor outcome. Through the use of COX-2 inhibitors, COX-2 could serve as a target for anticancer therapies with several molecular targets.[47]

Besides inducing angiogenesis, HIF-1 activates genes that are involved in increasing oxygen supply to peripheral tissues: the transactivation of erythropoietin, transferrin, the transferrin receptor, and heme oxygenase helps to elevate hemoglobin levels.

Invasion and Metastasis

Hypoxia can directly increase tumor cell invasiveness and metastasis.[4] In vitro studies have shown that hypoxia in combination with reoxygenation resulted in a dramatic but temporary increase in the metastatic potential of murine tumor cells.[48] Several hypoxia-responsive genes involved in the process of invasion and metastasis have been identified. Pennacchietti et al[49] showed that hypoxia sensitizes cells to the invasive qualities of hepatocyte growth factor by increasing the levels of the hepatocyte growth factor receptor, c-Met protooncogene. The hypoxic induction of met mRNA is activated by a cooperation between HIF-1 and AP-1 and stimulates growth, promotes shape changes, causes cell division, increases cell mobility, and produces proteases that lead to matrix degradation. These characteristics contribute to an invasive growth cascade that could result in metastatic spread of the hypoxic tumor.

Another potential contributor of the invasive process is the hypoxia-responsive plasminogen activator inhibitor-1 (PAI-1) gene.[46] Studies in PAI-1 knockout mice revealed that PAI-1 deficiency prevented local invasion and that by restoring the PAI-1 genotype the invasive phenotype was regained.[50] Another interesting candidate for hypoxia-induced metastasis and invasion is the urokinase plasminogen activator receptor (uPAR).[51] When bound by uPAR, it catalyzes the conversion of plasminogen to plasmin, thereby leading to the degradation of the extracellular matrix. Also, recently a group of extracellular matrix/adhesion molecules, including cathepsin D, fibronectin, uPAR, and matrix metalloproteinase 2, were reported to be hypoxia inducible, providing additional mechanisms by which the invasive cancer phenotype can be promoted in a hypoxic environment.[52]

In addition to local invasion, hypoxia may influence aspects of distant metastasis through the induction of the CXCR4 chemokine receptor. The chemokine receptor-ligand interaction is thought to govern some aspects of tissue-specific metastasis.[53] It was recently shown that VHL and hypoxia regulated the expression of CXCR4 in renal cancer, and renal cancer patients with high levels of CXCR4 expression had significantly poorer prognosis than the nonstaining.[54]

Endogenous Hypoxia Markers in Human Tumors

The measurement of tumor oxygenation is technically constrained in patients. Accessibility of the Eppendorf needle electrode to surface tumors or the need for administration of hypoxia marker drugs (EF5 or pimonidozole) followed by its immunologic detection has limited their common use. In theory, the most biologically significant measure of tumor hypoxia could be found in the expression of an endogenous hypoxia-induced gene product, either in the serum (for a secreted protein) or in biopsy specimens (intracellular proteins). Several groups are looking for such an independent prognostic marker.

However, the association between intratumoral pO_2 and the expression of endogenous hypoxia genes in human cancer is still somewhat unclear. Immunodetection of endogenous HIF-1 has been seen in a variety of human tumor types.[55] In lymph node-positive and -negative breast cancer, HIF-1 overexpression is associated with an unfavorable prognosis.[56] Similar data exist for early-stage invasive cervical cancer.[57] In contrast, it was shown that HIF-1 overexpression in squamous cell cancer of the head and neck was associated with improved survival.[58] Nevertheless, so far a clear spatial association of HIF-1 expression and tumor pO_2 has not been proven convincingly.

The only hypoxia-regulated marker genes whose expression has been shown to be dependent on intratumoral oxygenation status are Glut-1 and carbonic anhydrase CA IX.[59] Glut-1 correlated to intratumoral hypoxia measured by pimonidazole binding as well as Eppendorf needle electrodes in cervical cancer; moreover, absence of Glut-1 was associated with metastasis-free survival.[60] CA IX expression correlates positively to the level of intratumoral hypoxia measured in cervical cancer and is associated with poor survival in cervical cancer, breast cancer, and lung cancer.[61-63] In contrast, VEGF, the prototypic example of hypoxia-inducibility in vitro, did not show an association with the intratumoral oxygenation measured with the Eppendorf electrode in a recent clinical study.[64] These expression patterns suggest that multiple factors such as oncogenes, tumor suppressor genes, or growth factors may be regulating the expression patterns in vivo, not simply hypoxia.

The Therapeutic Advantage of Killing Cells That Express HIF-1

The concept of targeting HIF-1α to selectively kill or inhibit hypoxic tumor cells has now become feasible based on our understanding of this protein. In fact, some of the antitumor agents that are currently in clinical trial such as farnesyl transferase inhibitors, PI(3) kinase inhibitors, and TOR kinase inhibitors may act as antitumor agents in part through inhibiting HIF-1.[65] Inhibition of HIF-1 could lead to inhibition of tumor expansion by decreasing proangiogenic gene expression.[9] In addition to inhibiting the growth of hypoxic tumor cells, inhibition of HIF-1 could also inhibit the growth of tumor cells that possess oncogenic alterations in Ras, Src, or Her2/Neu or tumor cells that have lost the tumor suppressor genes VHL or PTEN.[66] Although the effect of loss of HIF-1α in tumors leads to inhibition of tumor growth, it does not eliminate tumors (F. Kaper and A. Giaccia, unpublished data, 2003). In fact, evidence from the literature indicates that inhibition of HIF-1 leads to a tumor growth delay, but eventually tumor growth resumes in a HIF-1–independent manner.[67] The advantage of targeting HIF-1 is its rapid response to changes in oxygenation, making it a good target for both transient (perfusion limited) and chronic (diffusion limited) hypoxic cells.

The concept, modeling, and development of potent hypoxia selective cytotoxins have indicated that the addition of such an agent to radiation can markedly potentiate cell killing.[68] Thus, the presence of HIF-1–activated cells in a tumor actually enhances the probability of gaining a therapeutic advantage with the use of a HIF-1–dependent cytotoxin. The premise that HIF-dependent cytotoxins could be an advantage with the use of fractionated radiotherapy is based on both the dynamic and static ways tumor hypoxia can occur by transient opening and closing of blood vessels and by metabolic consumption through successive cell layers surrounding a vessel, respectively.[68] Therefore, transient opening and closing of blood vessels and rehypoxiation after a fraction of radiotherapy can explain the pathophysiological changes in the tumor microenvironment that would provide the necessary microenvironment for selective HIF-1–dependent cytotoxins to be an advantage when combined with radiotherapy. In fact, it is also quite possible that HIF-1–dependent cytotoxins would be useful in targeting acutely hypoxic cells as HIF-1 becomes stabilized rapidly under hypoxic conditions.

These observations point toward a complex and fine-tuned regulation of hypoxia-responsive genes. Being exposed to spatial and temporal changes of hypoxia and reoxygenation, tumor cells go through an adaptive process. This response may lead to not only reversible but also irreversible hypoxia-induced changes. Thus, an understanding of the hypoxic cell phenotype may lead to a better understanding of the hypoxic tumor phenotype.

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Advances in Brief

Candidate Genes for the Hypoxic Tumor Phenotype[1]

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Abstract

In this study, we have analyzed changes induced by hypoxia at the transcriptional level of genes that could be responsible for a more aggressive phenotype. Using a series of DNA array membranes, we identified a group of hypoxia-induced genes that included plasminogen activator inhibitor-1 (*PAI-1*), insulin-like growth factor-binding protein 3 (*IGFBP-3*), endothelin-2, low-density lipoprotein receptor-related protein (*LRP*), BCL2-interacting killer (*BIK*), migration-inhibitory factor (*MIF*), matrix metalloproteinase-13 (*MMP-13*), fibroblast growth factor-3 (*FGF-3*), *GADD45*, and vascular endothelial growth factor (*VEGF*). The induction of each gene was confirmed by Northern blot analysis in two different squamous cell carcinoma-derived cell lines. We also analyzed the kinetics of PAI-1 induction by hypoxia in more detail because it is a secreted protein that may serve as a useful molecular marker of hypoxia. On exposure to hypoxia, there was a gradual increase in PAI-1 mRNA between 2 and 24 h of hypoxia followed by a rapid decay after 2 h of reoxygenation. PAI-1 levels were also measured in the serum of a small group of head and neck cancer patients and were found to correlate with the degree of tumor hypoxia found in these patients.

Introduction

Within solid tumors, hypoxia develops at distances beyond the diffusion capacity of oxygen from blood vessels (typically 100–150 μM; Ref. 1). In addition, hypoxia can develop in areas of a tumor with compromised blood flow due to aberrant vasculature formation and high interstitial pressure (2). The tumor microenvironment is a critical component that influences the behavior of transformed cells and their response to therapeutic interventions. Evidence from recent laboratory studies suggests that tumor hypoxia contributes to the progression of a more malignant phenotype by selecting for cells with a diminished apoptotic potential. Hypoxic conditions will also reversibly inhibit cell-cycle progression under certain growth conditions (3). Because cells exposed to low oxygen conditions are relatively resistant to conventional radiotherapy and chemotherapy, this population of cells significantly impacts clinical response to anticancer therapies.

Tumor hypoxia has been directly measured in a variety of human cancers including head and neck carcinomas, cervical carcinomas, and soft tissue sarcomas. Brizel *et al.* and Nordsmark *et al.* showed that, in head and neck carcinomas, hypoxia correlated with a lower probability of disease-free survival (4, 5) and that, in soft tissue sarcomas, hypoxia was associated with increased incidence of distant metastases (6). Hockel *et al.* (7) also found that hypoxia in cervical carcinomas resulted in increased local and distant failures. Interestingly, hypoxia predicted for distant failure not only in patients treated with radiotherapy but also in those treated with surgery alone. These studies suggest that hypoxia alters fundamental, physiologically important pathways that result in more aggressive tumor behavior in a wide variety of tumors.

We hypothesized that the development of an increased malignant phenotype can at least partially be attributed to changes in hypoxic gene expression. Under hypoxic conditions, the major transcription factor affecting gene regulation is HIF-1[3] (8). This factor regulates a diverse family of genes including VEGF (9), the urokinase receptor (10), tyrosine hydoxylase (11), endothelin 1 (12), nitric oxide synthase (13), erythropoietin (14), and numerous glycolytic enzymes (15). HIF-1 binds as a heterodimer consisting of an oxygen-sensitive HIF-1α (helix-loop-helix protein, HLH) subunit (16–18) and a constitutively expressed oxygen insensitive ARNT/HIF-1β (aryl hydrocarbon receptor nuclear translocator) subunit (17, 18). HIF-1α-deficient embryonic stem (ES) cells that are null at this locus fail to induce *HIF-1* target genes when exposed to hypoxia (19, 20). The HIF-1 heterodimer binds to a 6-bp [5′-ACGTG(C/G)-3′] hypoxia responsive element (HRE) that functions as a transcriptional enhancer in hypoxia-responsive genes (21). Although the majority of hypoxia-regulated genes are dependent on HIF-1, other transcription factors such as nuclear factor κB (22, 23), AP-1 (24, 25), and c/EBPβ (26, 27) as well as Egr-1 (28) are also activated by hypoxia.

We sought to characterize global transcriptional changes in tumor cells after exposure to hypoxic stress with the goal of determining how hypoxia influences the regulation of defined sets of genes involved in metabolic regulation, cell-cycle control, angiogenesis, and tissue invasion. We used cDNA array membranes containing 588 genes and compared gene expression under normoxic and hypoxic conditions in a squamous cell carcinoma-derived cell line. These studies resulted in the identification of nine hypoxia inducible genes that were subsequently confirmed by Northern blot analysis to be hypoxia-inducible.

To demonstrate the potential clinical applicability of hypoxic gene expression, we analyzed PAI-1 in the serum of patients with squamous cell carcinomas. Previous reports have suggested that PAI-1 plays a role in tissue invasion/remodeling and its up-regulation may contribute to the development of a more malignant tumor phenotype (29–31). Furthermore, increased expression of PAI-1 in some human tumors has been correlated with poor prognosis (32, 33). Most importantly, because it is a secreted protein, serum levels are readily detectable and may be useful as a molecular marker of hypoxia. We obtained serum samples from head and neck carcinoma patients and investigated whether PAI-1 levels correlated with the degree of tumor

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[3] The abbreviations used are: HIF-1, hypoxia-induced factor-1; EF-5, [2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide; PAI-1, plasminogen activator inhibitor-1; MIF, migration-inhibitory factor; BIK, BCL2 interacting killer; pO_2, partial pressure of oxygen; VEGF, vascular endothelial growth factor; DFO, desferroximine; IGFB-3, insulin-like growth factor-binding protein 3; Endo-2, endothelin-2; MMP-13, matrix metalloproteinase 13; LRP, low-density lipoprotein receptor-related protein; FGF-3, fibroblast growth factor 3.

hypoxia. The use of larger gene arrays may yield other secreted proteins and provide additional serum markers that reflect tumor hypoxia.

Materials and Methods

Cell Lines. Two cell lines obtained from American Type Culture Collection were used in this study. FaDu cells were established in 1968 from a punch biopsy derived from a hypopharyngeal tumor. The morphology of FaDu cells *in vitro* is epithelial. FaDu cells form well-differentiated epidermoid carcinomas when transplanted into immune deficient mice. SiHa cells were established in 1975 from tissue fragments derived from a squamous cell carcinoma of the cervix. The morphology of SiHa cells *in vitro* is epithelial. SiHa cells form poorly differentiated epidermoid carcinomas when transplanted into immune deficient mice. SiHa cells possess one to two copies of human papilloma virus type 16 integrated in their genomes and FaDu cells are human papilloma virus-negative. These two cell lines were chosen because they were both derived from squamous cell carcinomas, a tumor type in which hypoxia has been thought to be an important physiological modulator of malignant progression. Both of the cell lines were not used past 10 passages in cell culture.

Clontech Atlas cDNA Expression Array Membranes. Hybridizations were carried out according to the manufacturer's specifications. The membranes were prehybridized at 68°C for 30 min in ExpressHyb solution. Message RNA was purified by binding to a poly(A) column and probe that was generated by reverse transcription in the presence of [α-^{32}P]dATP. The membranes were then hybridized overnight with 0.5×10^6 cpm/ml probe at 68°C with continuous agitation. Membranes were washed twice with 2× SSC/1% SDS and twice with 0.1× SSC/0.5% SDS. All of the washes were carried out for 30 min at 68°C. The membranes were then visualized by phosphorimaging, and quantitation was performed with ImageQuant software. Counts were normalized to M_r 23,000 highly basic protein (Accession Number P40429) for loading controls.

Northern Blot Analysis. Total RNA was isolated with Trizol according to the manufacturer's protocol. RNA samples (10 μg) were denatured in glyoxal for 1 h at 50°C and separated by agarose gel electrophoresis. The gel was then transferred by capillary action overnight to Nytran membrane and cross-linked by exposure to UV light. Probes were generated by reverse trasnscription PCR using the manufacturer's primers (Clontech), gel-purified, and labeled with ^{32}P by random priming. Hybridization to ^{32}P-labeled probes was carried out at 65°C using ExpressHyb solution (Clontech) according to the manufacturer's protocol and washed for 2 h to a stringency of 0.2× SSC/1% SDS. Equal loading and transfer between lanes was demonstrated by methylene blue staining of 28S and 18S ribosomal bands before probing. All of the membranes were exposed by phosphorimaging and quantitated with ImageQuant software.

Hypoxic Treatment. FaDu and Siha cells were routinely cultured in DMEM + 10% FCS. Fresh media was exchanged 3–5 h before treating for varying amounts of time in a 37°C hypoxic incubator (Sheldon Manufacturing Inc.), which maintained an environment of less than 0.05% oxygen. The normoxic cells were maintained in a 37°C-incubator with 21% O_2. All of the experiments were performed at 70–80% cell confluency and the pH of the media remained between 7.0–7.4 for the duration of the experiment.

Immunohistochemical Staining of Tissues for EF-5 Binding; Photography and Analysis of Binding. The techniques used here were previously described (34, 35). For each patient, at least two tumor regions and two levels within each region (separated by 0.5 mm) were examined for regions of *in situ* EF-5 binding. The regions were imaged using a ×10 microscope objective (field size set electronically at 1.05×0.7 mm^2), and typically nine fields were examined for each section. To provide multiple pixels per cell while improving camera sensitivity, each image field consisted of 600×400 pixels each of which was a 2×2-bin of the actual camera chip pixels, with 12-bit gray-scale resolution.

Eppendorf pO_2 Histography and PAI-1 Determination. Eppendorf electrode measurements were taken through three tracks of neck nodes of patients with squamous cell carcinoma of the head and neck. Each pass with the probe recorded 50–100 measurements of oxygen concentration along the track. Measurements were also taken through one track of s.c. tissue of an uninvolved area in the neck to serve as a control. Serum levels of PAI-1 protein were measured using ELISA kits from biopool International (Ventura, CA) according to the instructions of the manufacturer. The PAI-1 ELISA has a detection limit of 0.5 ng/ml and measures latent (inactive) PAI-1, active PAI-1, and PAI-1 complexed with tPA/PAI and uPA/PAI. Using this assay and the manufacturer's protocol, the range of PAI-1 values found in individuals without pathophysiological conditions or in the third trimester of pregnancy is 4–43 ng/ml All of the human serum samples were obtained with the subjects' informed consent and were used for research purposes only. Total tumor burden (primary tumor and nodes) as assessed from computed tomography and magnetic resonance imaging scans indicated that there was no relationship between tumor burden and PAI-1 levels. Tumor burden ranged from 12.7 cm^2 to 60 cm^2. However, a relationship between median pO_2 values and PAI-1 levels in the serum was found. The graph represents data from eight patients with pathologically verified squamous cell carcinoma of the head and neck before any form of treatment.

Results

Fig. 1 shows a series of multiple gene array membranes that illustrate gene expression changes induced by hypoxia in a squamous cell carcinoma cell line (FaDu) originally derived from a pharyngeal wall tumor. These membranes were arrayed with 588 known genes categorized into six groups: (*a*) regulators of cell cycle; (*b*) apoptosis/tumor suppressors/oncogenes; (*c*) DNA damage/development; (*d*) cell

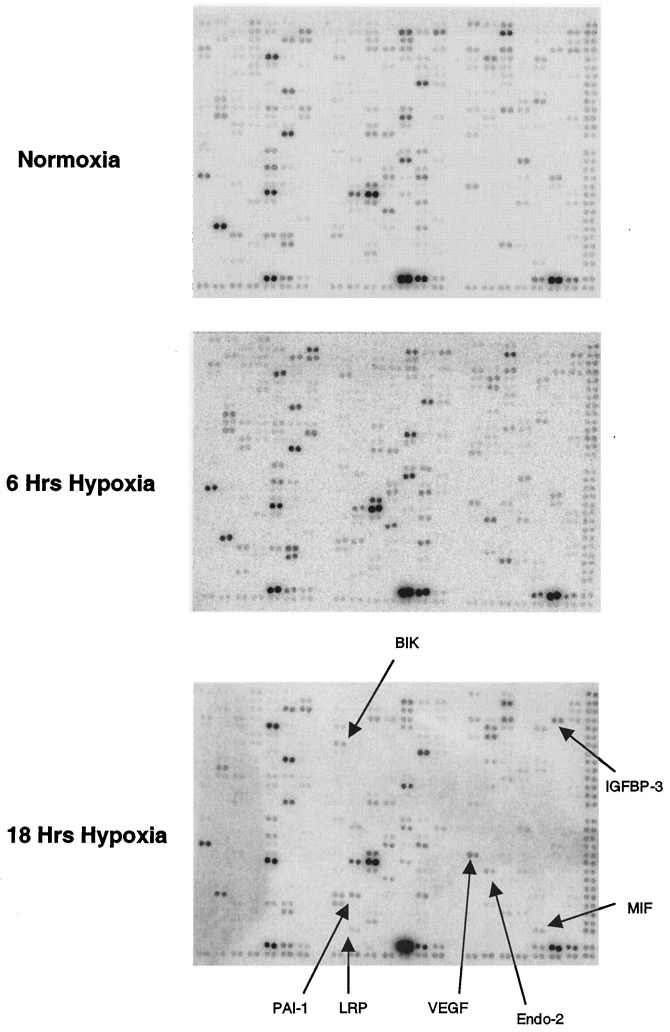

Fig. 1. Gene array membranes illustrating changes in gene expression induced by 6 and 18 h of hypoxia. Changes in the intensity of the target spot represent changes in the levels of mRNA expression in the hypoxia-treated samples as compared with the normoxic controls (*top panel*). The location of six hypoxia-inducible genes and VEGF are labeled in the 18 h hypoxia panel.

adhesion/angiogenesis; (*e*) regulators of invasion/cell-cell interaction; and (*f*) growth factors/cytokines. Cells were exposed to 6 or 18 h of hypoxia prior to mRNA isolation, and gene expression was then compared with cells cultured under normoxia. Quantitative analysis of these membranes was performed with ImageQuant software.

Because numerous investigators have shown that VEGF mRNA levels are exquisitely sensitive to hypoxia, we used this level of gene induction as a cutoff point for assessing hypoxia-induced genes. We only analyzed genes that demonstrated a greater level of induction than found with VEGF. Using this criteria, we identified nine genes (Table 1) that exhibited a greater than 3-fold induction under hypoxic conditions. Interestingly, the level of mRNA induction as determined by gene array analysis did not always correlate with the level of induction as determined by Northern blot analysis (Table 1). However, all of the genes that we initially identified based on their hypoxic inducibility when compared with VEGF were indeed found to be induced by hypoxia as assessed by Northern blotting (Table 1).

Fig. 2 is a composite of Northern blots that demonstrates the increase in mRNA expression of the seven most-hypoxia-inducible genes derived from membrane analysis. In this figure, we compared the induction of each gene in two different squamous cell carcinoma-derived cells. The kinetics of induction of each gene after exposure to 6 and 18 h of hypoxia was similar between FaDu cells and Siha cells (a cell line derived from a squamous cell carcinoma of the uterine cervix). In each Northern blot, a lane representing the effects of a 6-h treatment of cells with 100 μM DFO (an iron-chelating, hypoxia-mimetic agent) is also included for comparison (36). The consistency between the patterns of induction by hypoxia and DFO in these two different cell lines suggests that the regulation of these genes may be similar in squamous cell carcinomas, at least *in vitro*.

Table 1 *Analysis of candidate genes*

Gene	Accession number	Fold induction by array analysis	Fold induction by northern analysis
MIF	25639M	9.3	2 to 3
BIK	X89986	7.6	2 to 3
PAI-1	X04429	7.4	>10
Collagenase-3 (MMP13)	X75308	6.3	<2
LDL receptor-related protein	X13916	6.0	5 to 10
IGFBP-3	M31159	5.6	5 to 10
FGF-3 (INT-2)	X14445	5.6	<2
Endo-2	M65199	4.4	>10
GADD45	M65199	4.3	<2
VEGF	M32977	3.3	>10

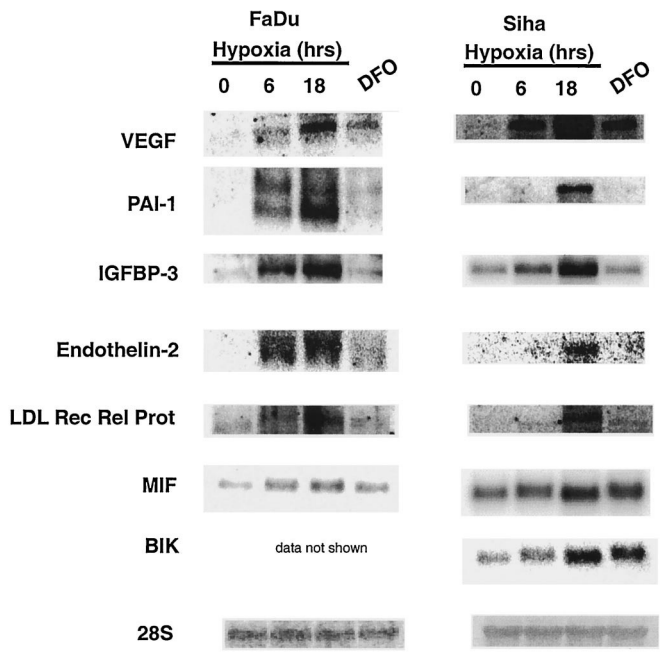

Fig. 2. Kinetics of *PAI-1*, *IGFBP-3*, *Endo-2* (*endothelin-2*), *LRP* (*LDL Rec Rel Protein*), *MIF*, *BIK*, and VEGF induction by hypoxia. The times for mRNA analysis were chosen so that membrane hybridization and Northern blotting could be directly compared. Northern blot analysis for two different squamous cell carcinoma cell lines, FaDu and Siha, is shown. In addition, the induction of each gene by the hypoxic mimetic agent DFO (6 h) is also included to further support the hypoxia-inducibility of each gene.

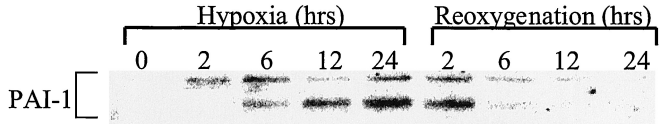

Fig. 3. Kinetics of PAI-1 mRNA induction by hypoxia and mRNA decay after reoxygenation in FaDu squamous carcinoma cells. PAI-1 mRNA is sensitive to changes in oxygen levels inasmuch as it exhibits a rapid increase under hypoxic conditions and decay on reoxygenation.

Fig. 3 shows an extended time course of PAI-1 induction in FaDu cells. Minimal PAI-1 mRNA is present under aerobic conditions, and an increase is seen 2 h after exposure of FaDu cells to hypoxia. These levels continue to increase for at least 24 h of hypoxia and return to near baseline levels after 6 h of reoxygenation. The changes in *PAI-1* gene expression were normalized to changes in *28S ribosome* gene expression which varied less than 10% in this experiment (data not shown). The sensitivity of *PAI-1* gene expression to levels of hypoxia makes it an ideal marker of *in vivo* hypoxia. In addition, because it is a secreted protein, serum levels can be monitored in a relatively noninvasive manner to determine the response to treatment and to detect early subclinical recurrence.

Fig. 4 is a comparison of PAI-1 protein levels and tumor hypoxia in serum from patients with squamous cell carcinomas. The patients are divided into two groups according to the degree of hypoxia found in their tumors. Hypoxia was determined either by immunohistochemical staining of EF-5 adducts, a nitroimidazole that is given by i.v. infusion in which it selectively and irreversibly binds to hypoxic cells (34, 35) or by intratumor Eppendorf pO_2 histography (37). In patients who received EF-5, PAI-1 levels were substantially greater in tumors that had EF-5 staining in more than 10% of the staining of the specimen (data not shown). However, because Eppendorf measurements are the most widely accepted method of estimating tumor oxygenation, we compared the levels of PAI-1 in the serum of oxic tumors with hypoxic tumors. PAI-1 values were plotted as a function of median pO_2 values (<4 mm Hg or >4 mm Hg) derived from Eppendorf readings. When compared with normal controls, there is a substantial increase in serum PAI-1 levels in both the low and high hypoxia groups and a trend toward higher PAI-1 levels in patients with more hypoxic tumors. This preliminary data on a small group of patients supports the initiative for a more extensive clinical analysis to investigate the relationship between PAI-1, tumor hypoxia, and outcome.

Discussion

When tumor cells are exposed to hypoxic stress, transcription of a discrete set of genes is initiated to serve a variety of cellular functions. It has been proposed that within solid tumors, hypoxia functions as a selective pressure leading to an apoptosis-resistant population of cells (38). In addition, other investigators have demonstrated that hypoxia increases the ability of tumor cells to metastasize (39, 40). These laboratory studies suggest that hypoxia influences tumor development by modulating gene transcription. This hypothesis is also supported by clinical data that correlates hypoxic tumors in soft tissue sarcomas,

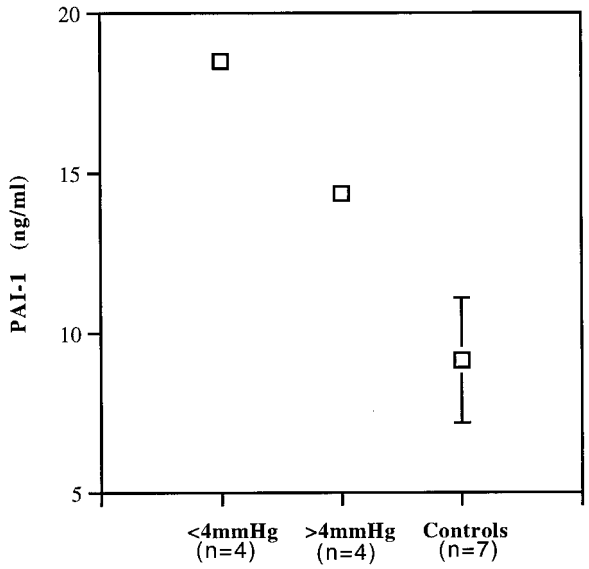

Fig. 4. Correlation of PAI-1 secreted protein levels in the serum of patients with tumor oxygenation. PAI-1 serum protein levels were detected by ELISA in triplicate. Tumor hypoxia was determined by Eppendorf histography. Patients were divided into two groups based on median pO_2 levels (>4 mm Hg or < 4 mm Hg). *Error bars*, two SEs.

head and neck carcinomas, and cervical carcinomas with worse overall survival (4–7). In this study, we sought to characterize gene expression changes that occur in response to hypoxia because we hypothesized that changes in gene expression might be responsible in part for the more aggressive phenotype of the hypoxic tumor cell.

With the use of a multiple gene array membrane, we screened 588 genes that had previously been identified to play a role in oncogenesis, for their response to changes in oxygenation. Because hypoxia had been previously shown to be a potent transcriptional activator of VEGF, we chose to use it as a cutoff point for identifying additional hypoxia-regulated genes. Using this criteria, we found nine genes that demonstrated greater hypoxic induction than VEGF as determined by ImageQuant analysis. The level of hypoxic induction when analyzed by Northern blot did not always correlate with the level of induction by array analysis because of differences in both the quantitative and qualitative aspects of probe and target gene hybridization. Such differences have been previously reported for p53-regulated genes (41). Furthermore, although the gene array screening was performed in FaDu cells, a similar level of induction was found by Northern blot analysis in Siha cells. It is noteworthy that Table 1 is not an exhaustive list of hypoxia-induced genes because the squamous carcinoma cells do not express or express at varying levels the genes on the array membrane. The gene array represents only a small fraction of expressed genes, and we analyzed only genes that were more-hypoxia-inducible than VEGF.

Table 1 is a ranked list of hypoxia-inducible genes that compares their induction by gene array analysis and Northern blot analysis. These genes can be broadly categorized into two groups: those involved in apoptosis (*BIK* and *IGFBP-3*) and those involved in local tissue/tumor response (*MIF*, *PAI-1*, *Endo-2*, *MMP-13*, *FGF-3*, *LRP*, and VEGF).

BIK and *IGFBP-3* are both proapoptotic genes (42, 43) that are transcriptionally up-regulated during hypoxia. Apoptosis is a complex process that reflects a shift in the delicate balance between pro- and antiapoptotic genes. During the time in which these genes are induced, we did not see any significant increase in apoptosis, which makes the function of these genes during hypoxia unclear. Perhaps other antiapoptotic pathways have become activated during hypoxia, which then negates the effects of these pro-apoptotic genes, or these genes may play other roles in growth regulation under hypoxic conditions.

The second and larger category of genes that we have identified by gene array analysis are involved in tissue remodeling and invasion. Young *et al.* have demonstrated that when tumor cells are exposed to hypoxia and reoxygenation, it results in an increased rate of metastasis as determined by lung colony formation of metastatic foci (39). Studies presented here and elsewhere suggest that many of the genes involved in the breakdown of the basement membrane and the eventual establishment of metastatic tumor foci are hypoxia-inducible (10, 44). Thus, the induction of tissue-remodeling genes by hypoxia undoubtedly contributes to the development of a more malignant phenotype.

A more detailed analysis of PAI-1 revealed that its regulation is exquisitely sensitive to hypoxia. Under normoxic conditions, there are undetectable levels of PAI-1 and between 2–24 h of hypoxia there is a gradual increase in PAI-1 mRNA. Reoxygenation of 2–6 h under normoxic conditions results in a marked decrease in PAI-1 expression to near-normoxic levels. Several groups have reported that PAI-1 is hypoxia-inducible in cell lines *in vitro* [(45, 46). Furthermore, analysis of the 5′ genomic sequence from the transcriptional start site of the *PAI-1* gene reveals a putative hypoxia responsive element (HRE) that provides a possible mechanism for PAI-1 regulation by hypoxia.[4]

As discussed above, increased PAI-1 staining of tumor sections has been correlated with a worse prognosis. However, the link between PAI-1, tumor hypoxia, and outcome has yet to be made. Because PAI-1 is a secreted protein, its serum levels can be easily measured and may serve as a surrogate marker of tumor hypoxia. Although we found a relationship between serum PAI-1 levels in head and neck cancer patients and the extent of hypoxia found in the tumors of these patients, a more thorough study is warranted to investigate whether other genes involved in plasminogen metabolism are also associated with tumor aggressiveness. It is also important to note that other pathophysiological conditions may elevate serum PAI-1 including pregnancy, cardiac ischemia, and blood clotting disorders, making a thorough clinical examination a necessity. In summary, PAI-1 represents but one hypoxia-regulated secreted protein that may eventually aid in cancer diagnosis, prognosis, and surveillance.

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Epigenetic Regulation of Gene Expression in Cervical Cancer Cells by the Tumor Microenvironment[1]

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ABSTRACT

Evidence is accumulating that the adverse tumor microenvironment both modifies the malignant progression of tumor cells and contributes to chemotherapy and radiation resistance. We hypothesized that some of the effects on malignant progression are mediated through the transcriptional regulation of genes responsive to the stresses of the microenvironment, such as low oxygen or low glucose conditions. To determine epigenetic changes in gene expression that were consistent with that hypothesis, we used an *in vitro* subtractive hybridization method, representational difference analysis, to identify hypoxia-induced cDNAs from cultured human cervical epithelial cells. We identified 12 induced genes: two novel genes (*HIG1* and *HIG2*), three genes known to be hypoxia-inducible (tissue factor, *GAPDH*, thioredoxin), and seven genes not previously identified as hypoxia-inducible [*HNRNP(a1)*, *ribosomal L7*, *annexin V*, *lipocortin 2*, *Ku(70)*, *PRPP synthase*, and *acetoacetyl-CoA thiolase*]. In cultured cells, *HIG1* and *HIG2* expression is induced by hypoxia and by glucose deprivation, but their expression is not induced by serum deprivation, UV, or ionizing radiation. The putative *HIG1* and *HIG2* open reading frames are expressed in cells, as confirmed by epitope tagging. In addition, tumor xenografts derived from human cervical cancer cells display increased expression of *HIG1* and *HIG2* when they are deprived of oxygen. Taken together, these data suggest a coordinated transcriptional response of eukaryotic cells to microenvironmental stresses found in the solid tumor.

INTRODUCTION

Tumor hypoxia is now being recognized as an independent prognostic indicator of poor patient survival in a number of tumor types (1–3), including squamous cell carcinoma of the uterine cervix (4). Interestingly, tumors with a low oxygen tension respond poorly to therapy regardless if they are treated by either chemotherapy or radiotherapy or even by surgery (4). Although radiation and some types of chemotherapy require oxygen to be maximally effective (5, 6), the fact that hypoxia predicted a worse outcome for the patients treated with surgery alone (4) implies that there is a fundamental biological difference in hypoxic tumors that cannot be explained by the effectiveness or access of the antitumor therapy. Additionally, model murine systems also identify a significant role for hypoxia-responsive genes in the growth of tumors *in vivo* (7, 8).

Hypoxia has also been shown to be a potent modulator of gene expression in a wide variety of cell lines tested *in vitro*. Specific protein accumulation (9, 10), gene induction (11), and gene repression (12) have been reported. Several transcription factors, AP-1 (13), NF-κB (14), and HIF-1[3] (11), have been identified whose activity increases under hypoxic conditions. HIF-1 is the transcription factor that responds most specifically and robustly to changes in oxygen concentration. This transcription factor is essential for development (8, 15) and is a heterodimer composed of a hypoxia-responsive HIF-1α subunit and a non-hypoxia responsive, constitutively expressed HIF-1β subunit (16). Under hypoxic conditions HIF-1α protein becomes stabilized (17) and binds with HIF-1β to an HRE containing the sequence 5′ACGTG(C/G)3′ (18). Although numerous HIF-1-responsive genes, such as *VEGF* (19), *glycolytic enzymes* (20), *urokinase receptor* (21), and *endothelin 1* (22), have been reported in the literature, it is still unclear whether any or all of these gene products are the underlying reason why hypoxic tumors are more aggressive.

We hypothesize that determining the identity of hypoxia-induced genes would be critical for understanding the mechanism(s) responsible for the more aggressive nature of tumors that contain regions of hypoxia. In this communication, we describe the use of the RDA technique to identify hypoxia-induced sequence tags. Because the RDA technology has many potential technical pitfalls, the identity of each tag and the corresponding gene was confirmed by sequencing, and the hypoxia inducibility of each tag was confirmed by Northern blot-

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[3] The abbreviations used are: HIF-1, hypoxia-inducible factor one; CMV, cytomegalovirus; EST, expressed sequence tag; FAA, flavone acetic acid; HA, hemaglutinin; HCE, human cervical epithelial cells; *HIG1* and *HIG2*, hypoxia-inducible gene 1 and gene 2, respectively; HPV, human papilloma virus; HRE, hypoxia-responsive element; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PBS-T, PBS-0.2% Tween 20; RDA, representational difference analysis; VEGF, vascular endothelial growth factor.

Table 1 Oligonucleotides used in this study

RDA OLIGOS	
Linker 1 (DS)	TTTTA**CCAGCTTATTCAATTCGGTCC**TCTCGCACAGGATGCATG
	ATGGTCGAATAAGTTAAGCCAGGAGAGCGTGTCCTAC
Primer 1 (SS)	CCAGCTTATTCAATTCGGTCC
Linker 2 (DS)	TTTTT**GTAGACATTCTAGTATCTCGT**CAAGTCGGAAGGATGCATG
	AACATCTGTAAGATCATAGAGCAGTTCAGCCTTCCTAC
Primer 2 (SS)	GTAGACATTCTAGTATCTCGT
HIG1	
hHIG1(for)	AATTTCTAGACGGAAGCCGGTTGGGGTGTGA
hHIG1(rev)	GGCTCTCGAGGACATGGGTCACTTACTTTAA
mHIG1cds(for)	CCGATCTAGAGGAAGGGACCCCGCGTCTCGGA
mHIG1cds(rev)	GGCGCTCGAGTCTAAACCCACATGTTATTTATTG
hHIG1HA(for)	CGATACGCGTAAGAAGAGATGCTGTCTTGG
hHIG1HA(rev)	CGATACGCGTCTACAGGCTGGCATAGTCAGGCACGTCATAAGGATAGCTAGGCTTAGGTTTTGCCC
HIG2	
mHIG2(for)	CCTTACTCCTGCACGACCTGG
mHIG2(rev)	GGCGCTCGAGCACATGTGCATTACACTGGAGA
hHIG2HA(for)	CGATACGCGTTAAGACCTCCTTCCA
hHIG2HA(rev)	CGATACGCGTTCACAGGCTGGCATAGTCAGGCACGTCATAAGGATAGCTCATGCTTCTGGATGG

ting. Using these rigorous criteria, the screen identified two novel expressed sequences that we cloned in their entirety; these two novel genes are termed *HIG1* and *HIG2*. *HIG1* and *HIG2* are also induced by the tumor microenvironmental stress of hypoglycemia, suggesting a coordinated response to both stresses. *HIG1* and *HIG2* may therefore represent a conserved mechanism for cells to respond to adverse microenvironmental stresses found within a tumor.

MATERIALS AND METHODS

Cell Lines and Tumor Formation. Normal human epithelial cells were immortalized *in vitro* by infection with retroviral constructs expressing HPV E6 and E7 oncoproteins. HCE.E6E7 were cultured in synthetic medium PFMR-4A (23). The cell lines SiHa, CaSki, and C33a, which were all derived from spontaneous human cervical cancers, were obtained from the ATCC and were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum. For stress treatments, cells were plated overnight and then treated the next day with either 256 nm UV at 1.2 J/m^2/s or gamma irradiation from a ^{137}Cs source at 3.8 Gy/min. Glucose and serum deprivation experiments were performed by washing the cells three times in PBS and replacing the indicated media (glucose-free RPMI with dialyzed serum or 0.1% fetal bovine serum RPMI). To generate tumor xenografts, 2.5–5 $\times$ 10^6 cells were injected s.c. into the flank of scid mice and allowed to grow into tumors that reached 1–2 cm in diameter before harvest. FAA (Lipha Chemical, New York, NY) was injected i.p. into the animals at 200 mg/kg in 5% sodium bicarbonate 24 h prior to tumor harvest.

Hypoxic Conditions. Cells were plated overnight in vented glass Petri dishes at 5 $\times$ 10^6 cells/100 mm dish. Hypoxic conditions were generated by placing the dishes in an anaerobic chamber (Sheldon Laboratories, Cornelius, OR) that was flushed with a gas mixture of 90% N_2, 5% CO_2, and 5% H_2. Any oxygen that was introduced into the chamber was consumed over a catalyst with hydrogen. A monitoring oxygen electrode was used to confirm an environment of 0.05% oxygen or less during experimentation.

RDA Technique. Briefly, the RDA technique (24) was performed on double-stranded cDNA that served as starting material for multiple rounds of *in vitro* subtraction and amplification. The cDNA was generated from mRNA isolated from control and 16-h hypoxia-treated HCE.E6E7 cells. The cDNA populations were digested with the restriction enzyme *Nla*III and ligated to different double-stranded linkers described in Table 1 (linker 1 and linker 2). The modified cDNA fragments were then individually amplified using a single primer corresponding to the linker sequence described in Table 1 (primer 1 and primer 2). The primer amplifying the driver population of fragments contained a 5′ biotin label. Three micrograms of biotinylated, driver cDNA and 0.1 μg of tester, nonbiotinylated cDNA were mixed together, lyophilized, resuspended in 2 μl of hybridization buffer (50 mM HEPES, pH 7.5, 10 mM EDTA, 1.5 M NaCl, and 2% SDS), covered in mineral oil, denatured at 95°C for 10 min, slowly cooled to 68°C over 1 h, and kept at 68°C for 4 more hours to allow hybridization. The hybridized cDNA populations were then diluted, mixed, and bound to 1 mg of M280 Dynal Strepavidin beads. The biotinylated DNA and any hybrid DNA was then removed with a magnet. The remaining, differentially expressed cDNA was reamplified using the tester primer, and then the subtraction was repeated three more times. The final cDNA population was digested with *Nla*III and cloned into the *Sph*I site of *pUC18* to generate the library of enriched fragments.

Northern Blotting and cDNA Isolation. Total RNA was isolated with TRIzol (Life Technologies, Inc., Grand Island, NY) following the directions of the manufacturer. Five to 10 μg of total RNA was denatured with glyoxal and size-fractionated on a 1% agarose phosphate gel. The gel was capillary-transferred to Hybond nylon (Schleicher and Shuell) and UV cross-linked. Probes were radiolabeled by random priming of gel-purified tag, full-length *HIG1*,[4]** or a fragment of *HIG2*

[4] The *HIG1* complete sequence can be found at NCBI GenBank accession no. AF145385 and *HIG2* at no. AF144755.

Table 2 Hypoxia-induced RDA tags

No. of hits	Accession no.	Gene	Response	Comment
106	AF145385	*HIG1*	HIG1	Novel
98	AF144755	*HIG2*	HIG2	Novel
48	J04038	*GAPDH*	HIG3	Known
11	X12671	*HNRNP*	HIG4	
11	U01691	*Annexin V*	HIG5	
8	S70154	*AcetoacetylCoA thiolase*	HIG6[a]	
7	X67698	*Tissue Factor*	HIG7	Known
7	X76388	*5-2A bp*	not induced	
6	Clone 68	*Unknown gene*	no signal	
5		*Alu-like*	not determined	
5	M14043	*Lipocortin 2*	HIG8	
5	X57959	*Ribosomal L7*	HIG9	
4	Clone 24	*unknown gene*	not induced	
3	X71490	*Vacuolar ATPase*	no signal	
3	D00860	*PRPP synthase*	HIG10	
3		*Alu-like*	not determined	
2	AF047441	*RNA poll 40Kd subunit*	not induced	
2	X77584	*thioredoxin*	HIG11	Known
2	U28386	*hSRP1(nuc loc)*	not induced	
2	J04611	*Ku(70)*	HIG12	
2	X85382	*Sm protein F*	not induced	
1		168 different clones		

[a] Minor 4.2-kb acetoacylCoA thiolase message is induced; response refers to mRNA changes by Northern blot analysis.

containing only the coding sequence in a *Stu*I fragment (Rediprime; Amersham, Arlington Heights, IL). Hybridization was carried out in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA at 56°C for *HIG1* and 65°C for *HIG2*, washed in 0.2–0.5× SSC at 56°C or 65°C, exposed to a phosphorimager plate, and visualized on a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA). A cDNA library constructed from mRNA purified from SiHa cells exposed to 16-h hypoxia was used to isolate full-length *HIG2*. This library was probed with radiolabeled *HIG2* tag using conventional methods. Full-length *HIG1* was isolated by first identifying overlapping ESTs from the NCBI human EST database, until a full-length sequence was generated (1.35 kb). PCR primers were then synthesized corresponding 5′ and 3′ UTRs to amplify the complete sequence using RT-PCR of SiHa RNA isolated after a 16-h hypoxia treatment. The full-length *HIG1* cDNA was then cloned and sequenced to confirm the predicted sequence. Recently, *HIG1* has also been identified by another group as *HSPC101*, a gene expressed in hematopoetic stem cells (25).

Construction of Epitope-Tagged *HIG1* and *HIG2*. *HIG1-HA* and *HIG2-HA* were constructed by reverse PCR. A (minus) primer was synthesized (Table 1, hHIG1HA[rev] or hHIG2HA[rev]) that hybridized to the carboxyl terminus of the ORFs. It extended the coding sequence by removing the endogenous stop codon, adding 36 nucleotides that code for 12 amino acids (the HA epitope), followed by a new stop codon, and ending with an *Mlu*I site. A second (plus) primer was synthesized (Table 1, hHIG1HA[for], or hHIG2HA[for]) that contained an *Mlu*I site, followed by a region that hybridized to the beginning of the 3′ untranslated region. The plasmid containing the cloned gene in an expression cassette (*pEGFPN1* with the *GFP* removed; Clontech) was then used as a template for PCR amplification using *pfu* polymerase (Stratagene). The full-length linear molecule containing the added sequences was then digested with *Mlu*I, ligated closed, and used to transform competent bacteria.

Immunological Detection of Epitope Tags. For immunohistochemical detection of HIG1HA and HIG2HA, cells were grown on chamber slides and were transfected with the indicated constructs using LipofectAMINE according to the instructions of the manufacturer (Life Technologies, Inc.). After 48 h the transfected cells were fixed in 2% paraformaldehyde, washed two times with PBS-T, blocked for 1 h in PBS-T with 3% BSA, and incubated for 1 h with anti-HA monoclonal antibody (Babco 101R) at 1:500 dilution in PBS-T with 3% BSA. The anti-HA treated slides were washed three times with PBS-T, incubated with fluoresceinated secondary goat anti-mouse antibody (Vector), also in PBS-T-BSA, washed three times in PBS-T, coverslipped with antifade solution (Vector), and visualized under epifluorescence using a Nikon microphot fluorescent microscope.

For immunoblot detection of HIG1HA and HIG2HA, protein extracts were generated from cell populations transiently transfected with the indicated expression plasmids. Cell populations were harvested and resuspended in PBS containing the protease inhibitor PMSF, 1.0 mM of the phosphatase inhibitor Na_3VO_4, and 1.0 mM of the kinase inhibitor NaF. Twenty-five micrograms of the extracts were electrophoresed on a 15% tricine gel and electrotransferred to the polyvinylidine difluoride membrane. The membranes were then blocked with PBS-T containing 5% milk for >1 h, incubated with anti-HA antibody at a 1:2000 dilution for 1 h in PBS-T milk, washed three times in PBS-T, incubated with horseradish peroxidase-conjugated goat antimouse antibody at a 1:2500 dilution in PBS-T milk, washed three times in PBS-T, and visualized with enhanced ECL (Amersham, Rockford, IL) on the Storm 860 (Molecular Dynamics).

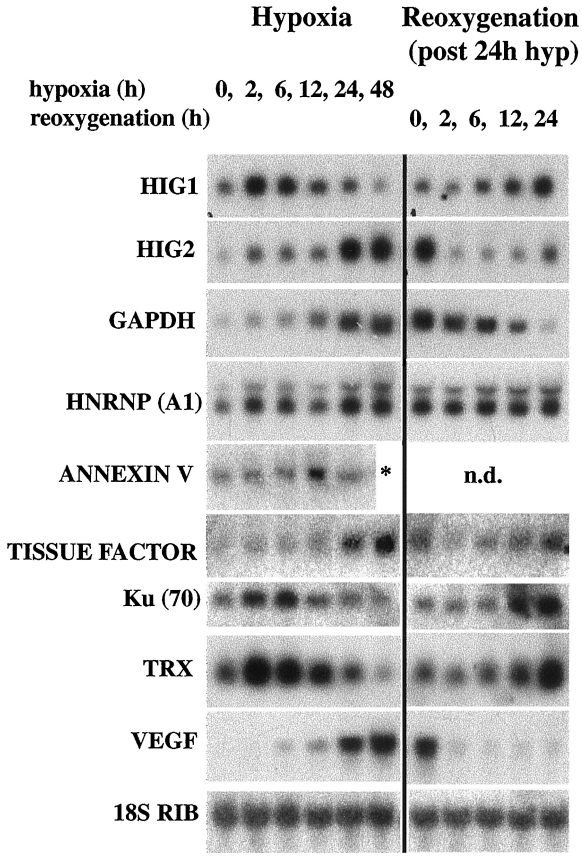

Fig. 1 Northern blot analysis of tags from the hypoxia-induced gene library. Northern blot analysis of RNA isolated from SiHa cells exposed to hypoxia with or without reoxygenation. Identical blots were probed with RDA tags corresponding to the indicated genes. Annexin V induction is shown from hypoxia-treated HCE. E6E7 cells. VEGF and 18S ribosomal probes were used as hypoxia-inducible gene expression and loading/transfer controls, respectively.

RESULTS

Human cervical epithelial cells stably immortalized with the HPV E6 and E7 oncoproteins served as the starting material for the construction of the RDA-enriched library. Four rounds of RDA subtraction of the oxic cDNAs from the hypoxic cDNAs generated a population of fragments representing genes that theoretically are induced by hypoxic treatment. Five hundred randomly chosen clones were partially sequenced, and these sequences were analyzed by NCBI BLAST to determine the frequency of each of the genes/ESTs in the enriched population. Because the most frequently repeated clones were unknown, we isolated full-length cDNAs that we decided to call *HIG1* and *HIG2*. We then reanalyzed the remaining unknown fragments from our 500 sequences against these complete genes, so that we could categorize all of the hypoxia-induced tags with respect to the two new unknown genes. The subtraction of hypoxic mRNAs from oxic mRNAs, designed to identify hypoxia repressed genes, was not performed because hypoxia globally reduces transcription and so it is more difficult to identify genes that are specifically repressed.

Because some of the genes are represented multiple times in this library, the 500 tags represent fragments of 21 genes that are present more than one time and 168 genes that are represented only once (Table 2). The two most frequently occurring genes are *HIG1* and *HIG2*. All of the clones represented more than one time that did not contain a highly repetitive element were tested by Northern blot for induction by hypoxia as well as by hypoxia and reoxygenation in SiHa cervical carcinoma cells. Representative Northern blot analysis is shown in Fig. 1 to demonstrate the kinetics of induction for each of the induced genes. It is interesting to note that approximately one third of the tags in Table 2 (7/19) were not induced, so it becomes clear why it is necessary to test each probe by Northern analysis. Although acetoacetyl-CoA thiolase sequence tag is listed as induced, the reported, major RNA (1.8 kb) for the gene does not change. However, there is a larger, hybridizing RNA species (4.2 kb) that is induced after 24–48-h hypoxia (data not shown). The Northern blot for annexin V is shown from HCE.E6E7 cells because annexin V is weakly induced in SiHa cells.

There are clearly distinct patterns of induction by hypoxia: one group of genes is induced to moderate levels with early kinetics (within 2–6 h), followed by a diminution (after 12 h), whereas another group of genes is induced to higher levels, but only after prolonged exposure to hypoxia (12–24 h). Interestingly, those genes that are induced by short-term hypoxia also seem to be induced by reoxygenation, [*e.g., HIG1*, *HNRNP(A1), Ku(70)*, and *thioredoxin*]. The genes listed in Table 1 that are not shown in Fig. 1 had only moderate hypoxic induction (2–3-fold). There are three genes identified in this group that have been previously reported to be hypoxia-inducible: *GAPDH* (26), *tissue factor* (27), and *thioredoxin* (28).

Because *HIG1* and *HIG2* represent two novel genes whose functions are unknown, we investigated these genes in more detail. We first examined the expression of *HIG1* and *HIG2* in a series of cervical cancer cell lines under oxic and hypoxic conditions *in vitro* (Fig. 2). Although *HIG1* is induced moderately within 2 h of hypoxia in all of the cell lines tested, it only remains elevated only in the SiHa cells. *HIG2* is more consistently induced from low basal levels in all of the cervical cancer cells tested. The major *HIG2* mRNA species is 1.4 kb in length, but there are two other mRNA species of minor abundance (8.0 and 9.0 kb) that are induced with identical kinetics to the major species (data not shown). The hypoxic induction of *HIG1* and *HIG2 in vivo* was also tested in tumor xenografts generated from the C33a cell line by Northern blot analysis of total tumor RNA. We compared untreated xenografts to xenografts that were made hypoxic by treatment of the host animal with FAA 24 h prior to explantation and RNA isolation (Fig. 2). We chose to examine expression changes after 24 h because this is the time shown to result in the most dramatic ablation of the tumor vasculature before causing extensive parenchymal necrosis. FAA treatment resulted in increased tumor hypoxia as measured by Eppendorf electrode (data not shown) and increased *HIG1* and *HIG2* expression by 1.2- and 2.4-fold, respectively. The moderate level of *HIG1* induction *in vivo* is not unexpected, because of the *in vitro* data. The portion of the human gene used as a probe in these experiments has low homology with mouse RNA and under the conditions used did not cross-hybridize.

The translated product of the putative ORFs from both genes is shown in Fig. 3. Both ORFs encode small peptides (93

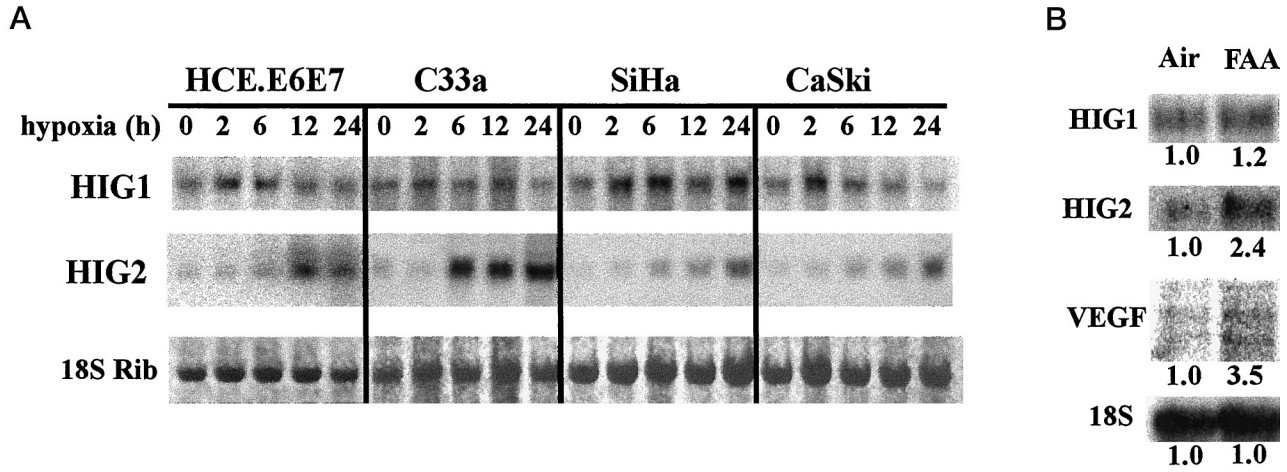

Fig. 2 Hypoxia inducibility of *HIG1* and *HIG2* in a series of cervical cell lines. *A*, Northern blot analysis of RNA isolated from HCE. E6E7 (normal cervical cells immortalized *in vitro*) and cervical tumor cell lines SiHa, CaSki, and C33a after treatment of the cells with hypoxia for the indicated times in hours. One nylon membrane was probed sequentially with *HIG1*, then *HIG2*. Methylene blue-stained 18S ribosomal bands were used as loading/transfer controls. *B*, Northern blot analysis of RNA isolated from C33a tumor xenografts either under control conditions (Air), or 24 h after treatment of host animals with FAA. *HIG1* and *HIG2* probes as indicated; VEGF and 18S ribosomal bands as hypoxia and loading controls, respectively.

HIG1 peptide sequence

MSTDTGVSLP SYEEDQGSKL IRKAKEAPFV PVGIAGFAAI VAYGLYKLKS
RGNTKMSIHL IHMRVAAQGF VVGAMTVGMG YSMYREFWAK PKP
Predicted MW 6951 Da

HIG2 peptide sequence

MKHVLNLYLL GVVLTLLSIF VRVMESLEGL LESPSPGTSW TTRSQLANTE
PTKGLPDHPS RSM
Predicted MW 10,144 Da

Fig. 3 Putative ORFs of *HIG1* and *HIG2*. ORFs were identified and translated from the full-length human *HIG1* and *HIG2* transcripts (GenBank accession nos. AF145385 and AF144755).

and 63 amino acid residues). Both peptide sequences were run through NCBI pBLAST, and no existing protein sequences of significant similarity were found. No functional motifs were identified in either sequence using world wide web-based search programs Prodom-Blast at Institut National de la Rescherche Agronomique[5] or Propsearch at European Molecular Biology Laboratory-Heidelberg.[6]

We next investigated whether *HIG1* and *HIG2* induction is unique to hypoxic stress or if it is elicited by other tumor microenvironment stresses, such as glucose deprivation or serum starvation, or by genotoxic stresses, such as UV or ionizing radiation. We also tested the hypoxia-mimetic, iron-chelating compound desferoxamine, which has been shown to induce expression from *HIF-1*-responsive genes (11). We chose the C33a cells for this series of experiments because these cells are the most responsive to glucose deprivation, which leads to good *HIG1* and *HIG2* induction (Fig. 4). Figure 5 shows Northern blot analysis of RNA isolated from C33a cells exposed to these stresses. *HIG1* is poorly responsive to hypoxic stress over this time course (Fig. 2). *HIG2* is induced strongly by hypoxia, the hypoxia-mimetic stress inducer deferoxamine, as well as glucose deprivation. UV light seemed to have little effect upon either *HIG1* or *HIG2* expression. In contrast, although ionizing radiation did not change *HIG1* expression levels, it did result in a moderate 2.5-fold induction of *HIG2* by 24 h. The similarities in the pattern of stress responsiveness of *HIG2* and that of the HIF-responsive *VEGF* gene suggest that *HIF-1* may be important in *HIG2* expression.

Finally, we determined if the ORFs that were identified in *HIG1* and *HIG2* were actually translated *in vivo*. To determine this, HA epitopes were added to the 3′ end of the putative ORF of both *HIG1* and *HIG2* by PCR, and the chimeras were expressed off of the CMV immediate early promoter after transient transfection into C33a cells. After 48 h, cells transfected with either CMVHIG1HA or CMVHIG2HA were fixed and incubated with anti-HA antibody to determine intracellular localization of the expressed product (Fig. 5*C*, *HIG1*; Fig. 5*F*, *HIG2*). Extracts of these cells were also examined by immunoblot for the protein size (Fig. 5*A, HIG1*, 5*D, HIG2*). Expression of a peptide is detected only in the cells transfected with plasmids expressing the tagged protein(s).

Immunoreactive material was detected in punctate pattern throughout the cytoplasm for *HIG1*, suggesting a vesicular or mitochondrial location. Immunoreactive material was found in a more diffuse, cytoplasmic localization for *HIG2*. It is difficult to draw too many conclusions from the cellular localization patterns of proteins that are overexpressed. Forced overexpression of some proteins can overwhelm normal trafficking patterns leading to aberrant localizations. Cellular morphology can be delineated by viewing Fig. 5, *B* and *D*, which represent the same fields seen in Fig. 5, *C* and *F*, but visualized under UV to excite the DAPI-stained nuclei. Because *HIG2* appeared on the immu-

[5] Address: http://www.protein.toulouse.inra.fr/prodom/doc/prodom.html.
[6] Address: http://www.embl-heidelberg.de/prs.html.

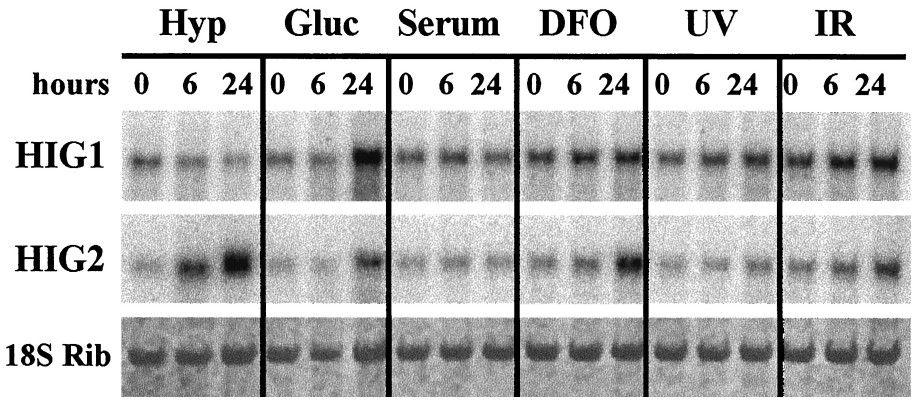

Fig. 4 Stress inducibility of *HIG1* and *HIG2* in C33a cells. Northern blot analysis of RNA isolated from C33a cells that were treated with hypoxia, glucose deprivation (0.0 mM glucose), serum deprivation (0.1%), UV light (20 J/m^2), or ionizing radiation (8 Gy). RNA was isolated at 6 and 24 h following treatments. The same membrane was probed sequentially with *HIG1* and *HIG2*; methylene blue stain of 18S rRNA for loading control.

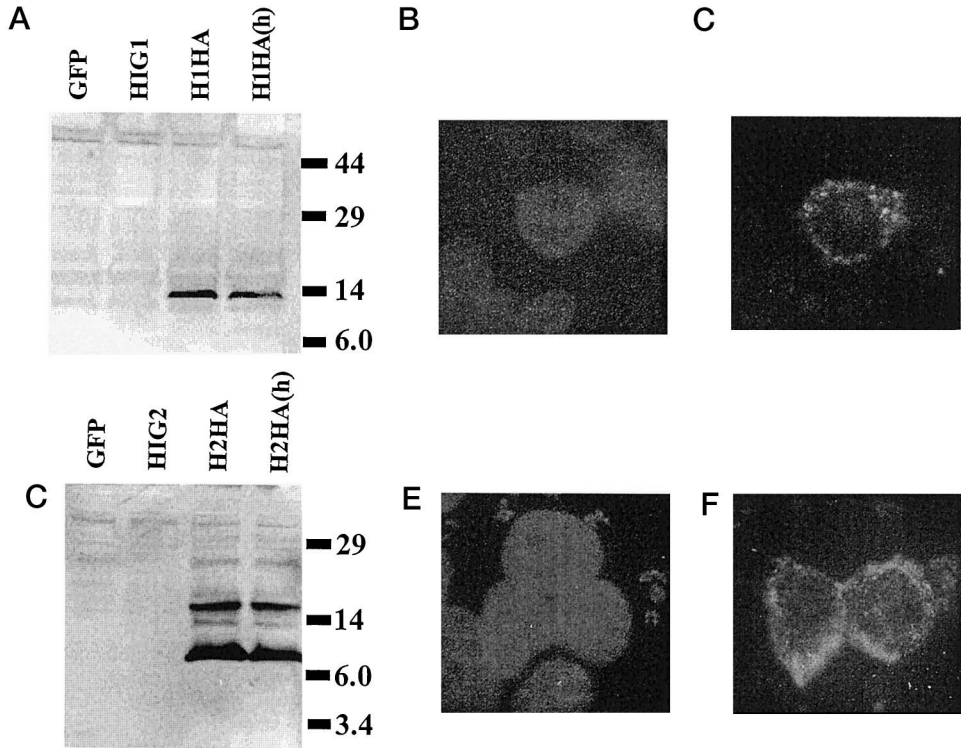

Fig. 5 Identification of immunoreactive material from epitope-tagged expression of HIG1HA (*A–C*) and HIG2HA (*D–F*). Immunoblot (*A*, *D*) of whole cell extracts from C33a cells transiently transfected with the indicated expression plasmids and probed with anti-HA monoclonal antibody (Babco 101R). Lanes labeled (h) indicate 6-h hypoxia treatment. Immunofluorescence of transfected cell population that was first fixed and then incubated with anti-HA antibody, and visualized with fluorescentated anti-mouse secondary antibody. *B*, HIG1HA cells visualized with nonspecific DAPI DNA stain; *C*, same field visualized with anti-HA antibody; *E*, HIG2HA cells visualized with DAPI stain; *F*, same field visualized with anti-HA antibody.

noblot as multiple bands, we hypothesize that this might represent multiple processed forms of the protein, producing altered migration. Interestingly, hypoxic treatment did not alter the protein quantity or electrophoretic mobility of the heterologously expressed proteins in C33a cell populations transfected with CMVHIG1HA or CMVHIG2HA and treated with hypoxia for 6 h (Fig. 5, *A* and *D*).

DISCUSSION

RDA analysis of HPV-immortalized cervical cells led to the identification of hypoxia-induced genes of several functional classes: genes involved in DNA metabolism, intermediate cellular metabolism, tissue structure, and angiogenesis. Two of the genes, *HIG1* and *HIG2*, are novel and have unknown function; three of the genes have already been reported to be hypoxia-inducible: *GAPDH* (26), *tissue factor* (27), and *THX* (28); and six are known genes that had not previously been shown to be hypoxia-inducible: *HNRNPA1*, *ribosomal protein L7*, *annexin V*, *lipocortin 2*, *Ku(70)*, *PRPP synthase*, and *acetoacetyl-CoA thiolase*. It is not yet clear if these genes are responsible for the more aggressive nature of hypoxic tumors, but *HIG1* and *HIG2* are shown here to increase in expression in tumor xenografts when they become more hypoxic (treatment with FAA). At present we can only speculate as to the function of these genes in the hypoxic tumor. Examining the regulation of expression of genes such as *HIG1* and *HIG2* in response to microenvironmental stresses yields insight into the epigenetic regulation of the hypoxic tumor phenotype. Coordinated expression of genes in

response to multiple microenvironmental stresses, such as hypoxia or glucose deprivation, leads to a cell that could be resistant to further stresses.

The *Ku(70)* gene product has been shown to participate in the repair of DNA double-strand breaks and could hypothetically respond to hypoxia-induced DNA damage (29) if it occurs. Another possibility is that hypoxia-stimulated *Ku(70)* expression could act as a tumor suppressor (30) as has been reported. Regardless of the reason, if low oxygen leads to *Ku(70)* induction, one side effect could be increased resistance to DNA-damaging agents or ionizing radiation.

Hypoxia has been shown to induce the expression of rate-limiting enzymes necessary for increased glycolysis in the absence of oxidative phosphorylation (20). One additional product of glycolysis is the generation of (reduced) NADH. The major generation of NADH during glycolysis is from the activity of GAPDH. The induction of GAPDH therefore serves two functions: (1) to contribute to increased glycolysis, and (2) to increase the production of reduced NADH. If hypoxic damage were mediated through a redox imbalance, then it would also be reasonable to induce a system for titrating the extra reducing/oxidizing equivalents. These extra reducing equivalents also could be used by several different cellular processes. For instance, it has been shown that thioredoxin can use NADH as a proton donor to activate the transcription factor AP-1 (31) or the estrogen receptor (32). To generate a large cellular pool of NADH, it might be necessary to synthesize more NAD. One of the precursors for NAD is adenine, and the rate-limiting step in the *de novo* generation of purines is PRPP synthase. Thus, PRPP synthase induction by hypoxia may be a physiological response to a redox imbalance.

Annexin V and *lipocortin 2* code for two family members of a group of cell surface calcium-binding proteins. Both molecules have been shown to play a role in the regulation of the fibrinolytic activity of plasmin (33). These gene products also decrease cell motility *in vitro* (34). Thus, as the cell surface properties of hypoxic cells could regulate adhesion and cell-cell connections in a tumor, the annexins, in concert with hypoxia-responsive proteases such as calpain (35), could impact tumor invasiveness and metastatic potential.

Wound healing, clot formation, and revascularization rely on delicately balanced factors, and one of the most potent inducers of angiogenesis is tissue hypoxia. It is not clear what the signaling mechanism is that leads to vessel generation, but many hypoxia-responsive genes are involved, such as *VEGF* (19) and *endothelin 1* (22). Hypothetically, hypoxia could act as a regulator of fibrinolysis by modification of plasmin activity through annexin induction (33). Additionally, tissue factor has been shown to regulate both the generation of fibrin (36) and the angiogenic activity of VEGF (37). Thus, tissue factor expression in response to hypoxia could be important in regulating the function of the vasculogenic factors of the tumor and as such could influence the growth rate of the tumor.

There are several reasons why we did not isolate all of the known hypoxia-inducible genes (such as *VEGF*) in this series of experiments. The primary reason is the nonrandom distribution of cleavage sites for the restriction enzyme *Nla*III used for digestion of cDNAs prior to linker ligation. Those genes, such as *VEGF*, with *Nla*III restriction sites organized in a nonrandom manner would generate fragments outside the 100- to 300-bp size that is most efficiently hybridized with the current protocol. The unlinked fragments, or the very large or very small fragments would be lost through the multiple rounds of subtraction and amplification. Another possibility is that additional *bona fide* hypoxia-inducible genes exist in the list of single hits from the 500 sequences. We chose to stop sequencing clones at 500 because we feel that this number gives good coverage to the complexity of our RDA library. We make this statement after comparing the diversity of the first 100 clones that were sequenced to the diversity of the next 400 clones that were sequenced.

Human *HIG2* has a high fraction of serine and threonine residues, 8 and 6 residues, respectively, of the total of 64 amino acid residues (Fig. 3). Serine 41 conforms to the consensus PKC phosphorylation site. Although *HIG2* migrates as a complex pattern of proteins, we could not detect a molecular weight shift of the protein with *in vitro* treatment of cellular extracts with nonspecific phosphatase (data not shown). It is therefore unclear what the multiple forms of the protein represent, although the major form of the expressed ORF does migrate with a mobility consistent with the predicted size. However, the modifications do not seem to be stress-inducible, and the uniform cytoplasmic distribution of the epitope-tagged *HIG2* does not provide clues to its function.

Taken together, these data support the hypothesis that there exists a cellular response to hypoxic stress that is regulated at the transcriptional level. This response is evolutionarily conserved in rodents and humans and consists of the coordinated regulation of many genes by *HIF-1*-dependent mechanisms as well as *HIF-1*-independent mechanisms so that the cell can survive in this adverse environment. The byproducts of this epigenetic response to low oxygen results in a tumor that is more resistant to conventional therapy and is more likely to invade or metastasize. The genes described in this report therefore represent potential new hypoxia-regulated proteins that can influence clinical outcome.

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Loss of *PTEN* facilitates HIF-1-mediated gene expression

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In glioblastoma-derived cell lines, *PTEN* does not significantly alter apoptotic sensitivity or cause complete inhibition of DNA synthesis. However, in these cell lines PTEN regulates hypoxia- and IGF-1-induced angiogenic gene expression by regulating Akt activation of HIF-1 activity. Restoration of wild-type *PTEN* to glioblastoma cell lines lacking functional PTEN ablates hypoxia and IGF-1 induction of HIF-1-regulated genes. In addition, Akt activation leads to HIF-1α stabilization, whereas PTEN attenuates hypoxia-mediated HIF-1α stabilization. We propose that loss of *PTEN* during malignant progression contributes to tumor expansion through the deregulation of Akt activity and HIF-1-regulated gene expression.

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The *PTEN* tumor suppressor gene was originally isolated from a homozygous deletion on human chromosome 10q23 in glioblastoma multiformes (GBMs) (Li et al. 1997; Steck et al. 1997). Germ-line mutations in *PTEN* result in autosomal dominant syndromes (Cowden disease, Bannayan–Zonana syndrome) associated with an elevated risk for cancer (Liaw et al. 1997; Marsh et al. 1997). *PTEN* encodes a dual-specificity phosphatase, and both somatic and germ-line mutations cluster within conserved regions of the phosphatase domain (Li et al. 1997; Liaw et al. 1997; Marsh et al. 1997; Steck et al. 1997). Surprisingly, physiological targets of PTEN were found to be lipid products of the PI(3)K proto-oncogene, PIP2(3,4) and PIP3(3,4,5) (Maehama and Dixon 1998). Taken together, PTEN negatively regulates downstream effectors of PI(3)K, an enzyme reported to affect multiple aspects of tumorigenesis (Fruman et al. 1998).

The proto-oncogene Akt is a Ser/Thr kinase that is a critical effector of PI(3)K and exhibits transforming capacity (Aoki et al. 1998). Akt activity is essential for transducing growth factor and integrin-mediated antiapoptotic effects (Datta et al. 1997; Khwaja et al. 1997). Several direct phosphorylation targets of Akt have been identified: Bad, GSK-3β, and Forkhead transcription factors (Datta et al. 1997; Pap and Cooper 1998; Brunet et al. 1999; Kops et al. 1999). In some cell types, Akt has been reported to modulate G_1 progression via inactivation of GSK-3β (Diehl et al. 1998). Additionally, Akt has been reported to mediate vascular endothelial growth factor (*VEGF*) induction under hypoxia (Mazure et al. 1997). Thus, Akt has multiple roles in tumorigenesis through the deregulation of cell cycle, enhancement of apoptotic resistance, and alteration of angiogenic potential.

Glioblastoma has one of the highest incidences of *PTEN* mutation (25%–60%) and *PTEN* mutation has been strongly associated with tumor differentiation (for review, see Cantley and Neel 1999). Importantly, low-grade gliomas rarely possess *PTEN* mutations, but, loss of heterozygosity (LOH) at 10q23 is found in ~70% of advanced glioblastoma (Cantley and Neel 1999). Occurrence of *PTEN* mutations late in tumorigenesis suggests that PTEN loss of function may provide a selective advantage for tumor expansion.

Previous studies have characterized the regulation of apoptotic sensitivity in genetically matched *PTEN* wild-type and null fibroblasts (Di Cristofano et al. 1998; Stambolic et al. 1998). As predicted, *PTEN* loss allows hyperactivation of the PI(3)K/Akt survival pathway and leads to increased apoptotic resistance (Davies et al. 1998; Haas-Kogan et al. 1998; Li et al. 1998; Stambolic et al. 1998). Glioblastomas are one of the most difficult tumors to treat as they are resistant to chemotherapy (Petersdorf et al. 1994) and are refractory to killing by many apoptotic stimuli (W. Zundel, unpubl.). Thus, apoptotic potential is one clear phenotypic manifestation thought to be conferred to cells on loss of *PTEN* function.

All tumors require angiogenesis for tumor expansion (Wesseling et al. 1997). Glioblastoma, in particular, is one of the most vascularized tumors and exhibits increased expression of many proangiogenic genes such as *VEGF* and fibroblast growth factor (FGF) (Wesseling et al. 1997). Because PI(3)K and Akt previously have been implicated in the induction of *VEGF* expression by hypoxia (Mazure et al. 1997), we hypothesized that wild-type *PTEN* expression in *PTEN* mutant glioblastoma cell lines could alter the cellular response to hypoxia and subsequent *VEGF* expression.

Results

PTEN *expression attenuates hypoxia-mediated activation of Akt*

Hypoxia and growth factors (e.g., IGF-1, insulin, PDGF)

[*Key Words*: *PTEN*; angiogenesis; glioblastoma; gene expression]
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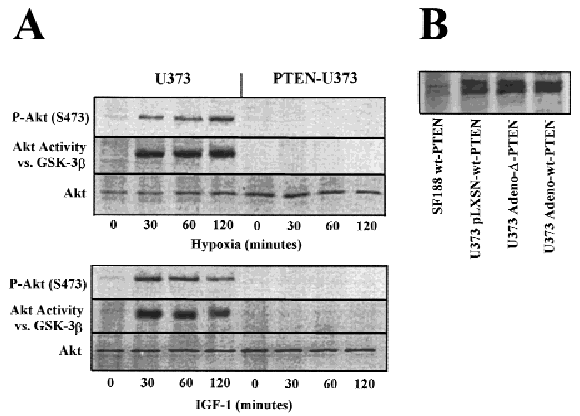

Figure 1. Hypoxia induces Akt activity that is regulated by PTEN. (*A*) U373 cells were retrovirally infected with wild-type *PTEN* and subjected to hypoxia or IGF-1 (50 ng/ml) for the indicated times. Cells were harvested and the lysates allocated for immunoblotting using α-Phospho-Akt (S473) (*top*) α-Akt antibodies or Akt kinase activity using GST–GSK-3β as a substrate. (*B*) U373 cells were either retrovirally (wt–*PTEN*) or adenovirally (wt or phosphatase inactive-Δ *PTEN*) infected. The U373-infected cells or a glioblastoma cell line possessing two wt–*PTEN* alleles (SF188) as control were immunoprecipitated and immunoblotted with Santa Cruz SC-571 and UBI-ID 07016, respectively.

are critical modulators of tumor angiogenesis (Warren et al. 1996; Mazure et al. 1997; Zelzer et al. 1998; Wang et al. 1999). If these stimuli activate proangiogenic gene expression through a PI(3)K/Akt-dependent pathway (Fruman et al. 1998), *PTEN* should block gene expression by inhibiting Akt activation in response to hypoxia. To test this hypothesis, the *PTEN* mutant glioblastoma cell line U373 was used to assess the effects of wild-type *PTEN* expression on hypoxia- and IGF-1-stimulated Akt phosphorylation and kinase activity (Fig. 1A). Hypoxia and IGF-1 stimulated Akt phosphorylation on Ser-473 and Akt kinase activity toward a GST–GSK-3β substrate within 30 min, and that activity remained sustained for >2 hr. Expression of wild-type *PTEN* in the same cells completely blocked hypoxia- and IGF-1-induced Akt phosphorylation and kinase activity, consistent with the inhibition of Akt by wild-type PTEN in serum-stimulated glioblastomas (Haas-Kogan et al. 1998).

We next assayed PTEN levels 72 hr postinfection (Fig. 1B). Viral-mediated gene transfer into U373 yielded ~11%–22%-increase in PTEN levels. Retroviral infection yielded ~85%–94% infectivity whereas the adenoviral-mediated GFP–*PTEN* infection efficiency was 52%–90%.

PTEN *overexpression does not alter serum deprivation or hypoxia-mediated apoptosis and only partially alters DNA synthesis*

To evaluate the role of *PTEN* expression on DNA synthesis we analyzed [^{3}H]-thymidine incorporation in serum-deprived or mitogen-stimulated U373 cells (Fig. 2A). Wild-type *PTEN*, but not phosphatase-inactive *PTEN*, reduced DNA synthesis by ~60%. Although significant, DNA synthesis was not completely attenuated and the *PTEN*-infected cells did survive and replicate. Although these cells were under selection for *PTEN* expression, it is possible that the DNA synthesis observed is mediated by 10%–40% of uninfected cells. Hypoxia completely inhibited DNA synthesis in U373 and *PTEN*-expressing U373, whether in the presence or absence of serum, indicating that hypoxia-mediated cell cycle arrest is dominant even in cells possessing deregulated and active Akt.

Because the role of PI(3)K and Akt in anti-apoptotic functions is based on developmental models and transformed or immortalized cell lines, we evaluated the sensitivity of U373 to the proapoptotic stimuli of serum deprivation and hypoxia (Fig. 2B). We found that U373 and other glioblastoma cell lines are extremely resistant to many apoptotic stimuli (data not shown). However, expression of *PTEN* even at highly elevated levels (Fig. 1B) fails to alter apoptotic sensitivity of U373 cells to serum deprivation or hypoxia at 72 hr, despite having no detectable Akt activity (Fig. 1A). Other apoptotic stimuli,

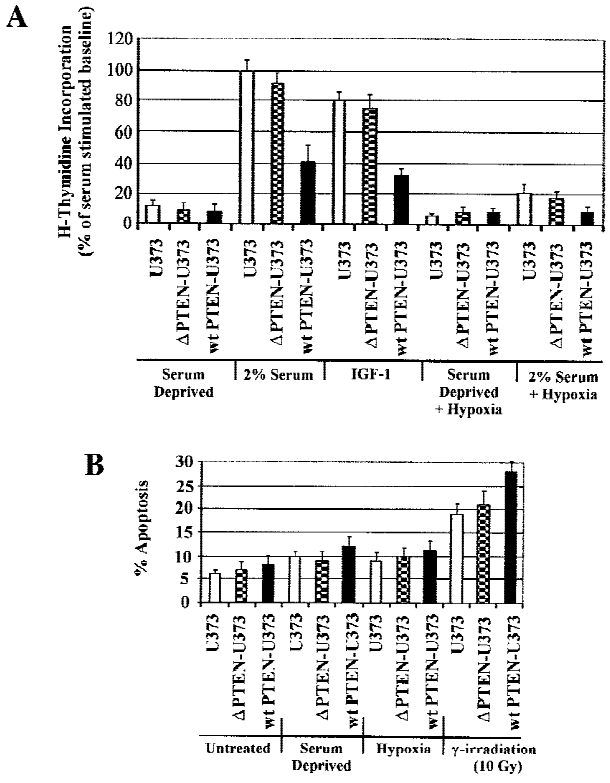

Figure 2. *PTEN* overexpression incompletely inhibits DNA synthesis and has minimal effects on apoptosis. U373 cells were infected with adenovirus containing wild-type or phosphatase inactive-Δ *PTEN*. (*A*) Forty-eight hours postinfection, serum-starved U373-infected cells were labeled with [^{3}H]-thymidine, treated as indicated for 24 hr, and assayed for [^{3}H]-thymidine incorporation. (*B*) Forty-eight hours postinfection, U373-infected cells were treated as indicated and assayed for apoptosis.

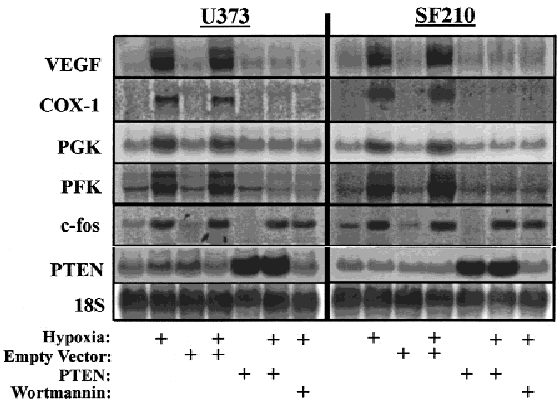

Figure 3. PTEN regulates the expression of HIF-1-regulated genes under hypoxia. U373 or SF210 cells were retrovirally infected with wild-type *PTEN* or empty vector control. Thirty-six hours postinfection, U373 and SF210 cells expressing *PTEN* or empty vector control and parental cells ± 100 nM wortmannin were subjected to 9 hr of hypoxia. mRNA was isolated and analyzed by Northern blot using *VEGF*, *COX-1*, *PGK-1*, *PFK*, c-*fos*, and *PTEN* probes.

such as γ-irradiation, only minimally sensitized glioblastoma cells to apoptosis by *PTEN* expression. These results suggest that glioblastomas have acquired anti-apoptotic mutations other than loss of *PTEN* that are sufficient to protect the cell from some physiological insults. Figure 2A and B, indicates that loss of *PTEN* function facilitates tumor expansion in a manner independent from its anti-apoptotic and -proliferative activities.

PTEN *restoration regulates expression of endogenous hypoxia-inducible genes*

To investigate whether *PTEN* regulates endogenous expression of hypoxia-induced genes implicated in angiogenesis, we analyzed *VEGF* and *COX-1* mRNA expression in hypoxia-treated U373 and SF210 (Fig. 3). The expression levels of other hypoxia-inducible genes required for glycolysis (*PGK-1*, *PFK*) were also analyzed. Wild-type *PTEN* expression and the PI(3)K inhibitor wortmannin both blocked endogenous *VEGF*, *COX-1*, *PGK-1*, and *PFK* induction in response to hypoxia when compared with empty vector controls. This effect of PTEN is not due to global down-regulation of transcription as seen by the ribosomal 18S panel. Furthermore, the negative regulation of PTEN on hypoxia-inducible genes is specific to the PI(3)K/PTEN/Akt/HIF pathway because hypoxia-induced c-*fos* mRNA levels (which are regulated by the MAPK pathway) are insensitive to PTEN expression. These results clearly establish a role for *PTEN* in the regulation of not just angiogenic factor expression but hypoxia-inducible genes in general.

PI(3)K, Akt, and HIF-1α are required for VEGF *expression that is negatively regulated by wild-type* PTEN

Figure 3 illustrates that wild-type *PTEN* inhibits various hypoxia-inducible genes shown previously to be dependent on hypoxia-inducible factor 1 (HIF-1) (Semenza 1998). HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β/ARNT subunit and a hypoxia-stabilized HIF-1α subunit (Semenza 1998). Normally, HIF-1α is expressed constitutively but rapidly degraded under oxic conditions by ubiquitin-mediated degradation (Semenza 1998). To evaluate the role of PI(3)K and Akt in HIF-1α-dependent transactivation, we used a reporter construct containing five tandem repeats of the hypoxia-responsive element (*HRE*) from the *erythropoietin* gene, which has been shown previously to be HIF-1 responsive (Semenza et al. 1991; Mazure et al. 1997). Similar patterns of VEGF and HRE reporter activity were induced by hypoxic exposure and inhibited by wild-type PTEN expression (Fig. 4A). We also found that constitutively active PI(3)K (p110*) and Akt (myr–Akt) were sufficient to transactivate both *VEGF* and *HRE* reporter constructs to levels comparable to IGF-1 stimulation (Fig. 4A). However, whereas the p110* reporter stimulation could be attenuated by PTEN, myr–Akt could not. It has been reported previously that myr–Akt is unrespon-

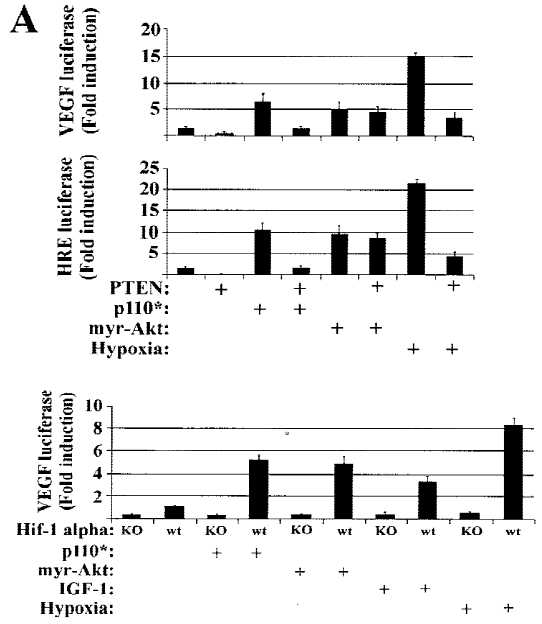

Figure 4. PTEN regulates PI(3)K-induced *VEGF* expression upstream of Akt in a HIF-1α dependent fashion. (*A*) U373 cells were cotransfected with either a VEGF–luciferase (*top*) or a HRE–luciferase (*bottom*) reporter in combination with either a constitutively active PI(3)K (*p110**) or a constitutively active Akt (*myr–Akt*). Portions of the parental U373 and the *p110** and *myr–Akt*-transfected cells were then retrovirally infected with *PTEN*. Thirty-six hours postinfection, the cells were treated under oxic or hypoxic conditions for 12 hr, followed by lysis and luciferase activity quantitation. (*B*) *HIF-1α* homozygous null and HIF-1α wild-type MEFs were cotransfected with VEGF–luciferase in combination with either a constitutively active PI(3)K (*p110**) or a constitutively active Akt (*myr–Akt*). Portions of these cells were then retrovirally infected with *PTEN*. Thirty-six hours postinfection, the cells were treated with IGF-1 or exposed to hypoxic conditions for 12 hr followed by lysis and luciferase activity quantitation.

sive to *PTEN* expression (Li et al. 1998), supporting the concept that PIP(3,4)/PIP(3,4,5) binding serves primarily as a localization signal (Kohn et al. 1996).

To genetically link hypoxia and IGF-1 activation of PI(3)K/Akt to HIF-mediated transactivation, we studied the ability of the PI(3)K/Akt pathway to modulate *VEGF* reporter activity in mouse embryo fibroblasts (MEFs) derived from homozygous null *HIF-1α* mice (Ryan et al. 1998). Hypoxia, IGF-1, and constitutively active forms of PI(3)K and Akt were all dependent on HIF-1α for *VEGF* reporter transactivation (Fig. 4B). Thus, hypoxia and IGF-1 stimulate PI(3)K and Akt, which leads to HIF-1α-dependent *VEGF* transactivation.

PTEN and Akt mediate degradation/stabilization of HIF-1α

Under low oxygen conditions, HIF-1α is stabilized by an undetermined mechanism that is necessary for translocation, heterodimerization, and transactivation (Semenza 1998). To determine whether *PTEN* expression altered the stability of HIF-1α PTEN-infected cells were exposed to hypoxic challenge for various periods of time (Fig. 5A). PTEN completely suppressed the stabilization of HIF-1α protein by hypoxia, indicating a possible role for PI(3)K stabilization of HIF-1α (Fig. 5A). To explore the role of Akt in HIF-1α stabilization, we infected cells with an inducible constitutively active form of Akt (myr–Akt–ER) or an inactive control (myr–A2–ER) under oxic conditions (Fig. 5B). Activation of myr–Akt–ER by 4-hydroxytamoxifen (4-HT) resulted in stabilization of HIF-1α, whereas activated myr–A2–ER had no effect. These results strongly indicate that hypoxia mediates HIF-1α stabilization through an Akt-dependent pathway in glioblastoma cells.

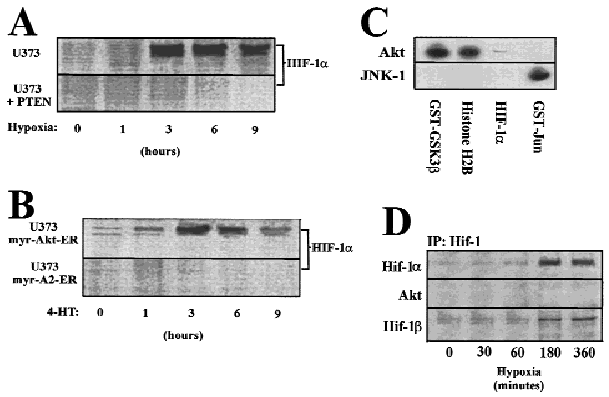

Figure 5. Akt stimulates HIF-1α stabilization. (*A*) U373 cells were retrovirally infected with wild-type PTEN. Thirty-six hours postinfection, U373 parental cells and *PTEN*-expressing U373 were subjected to hypoxia for the indicated time. Cells were lysed at the indicated times followed by SDS-PAGE, transfer, and immunoblotting with anti-HIF-1α. (*B*) Cells were retrovirally infected with *myr–Akt–ER* or *myr–A2–ER*. Thirty-six hours postinfection, the cells were subjected to induction by 4-HT for the indicated time and analyzed as in *A*. (*C*) U373 cells were serum-deprived and subjected to 1 hr of hypoxia to activate Akt or to UV-C (10 J/m^2) for JNK-1 activation for use in immune complex kinase assays. Reactions were performed using 500 ng of either histone H2B, GST–jun, GST–GSK-3β, or HIF-1α as substrates and Akt or JNK-1 immunoprecipitations for in vitro kinase assays. Kinase reactions were subjected to SDS-PAGE, and gels were dried and visualized by PhosphorImaging. (*D*) Cells were subjected to hypoxia for the indicated times, lysed, immunoprecipitated using anti-HIF-1α, and subjected to SDS-PAGE, transfer, and immunoblot using anti-HIF-1α anti-Akt, or anti-HIF-1β antibodies.

Akt phosphorylates proteins containing a RXRXXS/T consensus (Datta et al. 1997; Pap and Cooper 1998; Brunet et al. 1999; Kops et al. 1999). HIF-1α does not contain this motif or any significant amino acid sequence having a similar pattern. To confirm that Akt-induced HIF-1α stabilization and transactivation is not a result of direct phosphorylation of HIF-1α, we used HIF-1α as a substrate in in vitro kinase assays (Fig. 5C). HIF-1α was a poor substrate for Akt compared with known Akt substrates GSK-3β and histone H2B. JNK-1 kinase activity is included as a negative control to confirm the specificity of Akt kinase activity. These results suggest that Akt modulates an undetermined downstream effector that regulates HIF-1α stabilization.

Often signaling complexes are formed that allow for the identification of pathway components. We immunoprecipitated HIF-1α from hypoxia-stimulated cells at various time points to detect any possible Akt and HIF-1α complex formation (Fig. 5D). No detectable Akt/HIF-1α interaction was detectable, whereas HIF-1α and HIF-1β complexes formed as expected. Although it is possible that Akt and HIF-1α interactions are too transitory or labile to be detected, the lack of an Akt phosphorylation site, coupled with no significant in vitro phosphorylation, strongly suggests that Akt is modulating other protein(s) that increase HIF-1α stabilization.

Discussion

In this study we observed that *PTEN* inactivation leads to hypoxia-inducible gene expression in glioblastoma lines irrespective of PTEN's effects on apoptosis and cell cycle control. We have shown that PTEN can regulate hypoxia- and growth-factor stimulated transcription of VEGF and HRE promoters. Interestingly, stimulation of Akt by growth factors, p110*, or expression of myr–Akt generated approximately one-fifth to one-third of the reporter response compared with hypoxia. This suggests that hypoxia potentiates the transactivation of HIF-regulated genes by other mechanisms in addition to Akt. We have shown additionally that HIF-1α is not a direct substrate for Akt but that HIF-1α stabilization is signaled through Akt. Cumulatively, these findings suggest a new role for *PTEN* mutations in the regulation of hypoxia-inducible gene expression and give insight into why these mutations are observed predominantly in the late stages of tumor development.

Although multiple studies have implicated a role of PTEN in apoptosis through Akt, apoptosis and angiogenesis need not be mutually exclusive events. As tumor growth exceeds vascular density, the tumor develops

nonvascularized areas in which metabolic byproducts, acidosis, low growth factor and nutrients, as well as hypoxia, stimulate apoptosis (Yuan and Glazer 1998). Thus, apoptosis driven by the tumor microenvironment could potentially select for loss of negative regulators of apoptosis, such as *PTEN*, as has been shown for other tumor suppressors that regulate apoptosis, such as *p53* (Graeber et al. 1996). Therefore, loss of *PTEN* would both increase cell survival in an adverse tumor microenvironment and increase responsiveness to hypoxia-induced HIF-1 activity that would stimulate angiogenic gene expression as well as other essential genes required to survive under low oxygen conditions. This study suggests that glioblastoma cell lines are very apoptotically resistant in a *PTEN*-independent fashion, further inferring alterations in apoptotic genes irrespective of *PTEN* loss and provides alternative explanations for the importance of *PTEN* mutations in tumor expansion.

PTEN is not the only tumor suppressor gene implicated in HIF-1α regulation. The tumor suppressor protein Von-Hippel–Lindau (VHL) also regulates HIF-1α expression by modulating its protein stability, presumably via its E3 ubiquitin ligase activity (Iwai et al. 1999; Maxwell et al. 1999). Tumor cells that have mutant forms of *VHL* exhibit increased expression of many HIF-regulated genes under aerobic conditions. In contrast, *PTEN* mutations have minimal effect on oxic expression of HIF-regulated genes but potentiate their induction following hypoxia or growth factor stimulation.

The frequency of *PTEN* mutation underscores how the most frequently mutated genes in cancer often control multiple facets of tumorigenesis. For instance, *p53* is implicated in apoptosis, cell cycle control, and genomic instability (Giaccia and Kastan 1998), whereas *ras* is implicated in cell cycle, apoptosis, and angiogenesis (Campbell et al. 1998). In contrast, the ultimate effectors of apoptosis, the caspases, are infrequently mutated in cancers (Mandruzzato et al. 1997). *PTEN* mutations result in a well-documented deregulation of critical lipid second messengers that control pivotal steps in pathways that suppress apoptosis, increase proliferation, and stimulate angiogenesis. Thus, the finding that wild-type PTEN controls multiple avenues of tumor function makes it a likely target for therapeutic intervention in tumors such as glioblastoma or prostate cancer in which gene therapy approaches are feasible.

Materials and methods

Cell culture and reagents

U373, SF188, and SF210 glioblastoma cell lines were maintained in DMEM containing 10% (vol/vol) FBS (GIBCO BRL). All experiments were performed at 80%–100% confluence. The *HIF-1α* nullizygous and parental cell lines (Ryan et al. 1998) were maintained in DMEM containing 15% (vol/vol) FBS. IGF-1 (GIBCO BRL) and wortmannin (Biomol) were prepared as 1000× stock solutions. The antibodies used were Akt and Akt/Ser-473 (New England Biolabs no. 9272 and 9271, respectively), JNK-1 (Santa Cruz SC-571), PTEN (Santa Cruz SC-571, UBI-ID 07016), and HIF-1α and HIF-1β (Transduction Labs. H72320 and A78420, respectively).

Apoptosis

Apoptosis was quantified as described previously (Graeber et al. 1996). Briefly, following treatment, cells were incubated with 2 μg/ml each of bis-benzamide (Hoechst no. 33342, Sigma) and propidium iodide (Sigma) for 15 min. Viability ratios (number of apoptotic cells/total number of cells) were determined by scoring low-magnification fields of randomly selected fields for cells with condensed and fragmented nuclei and loss of membrane integrity. Fields of cells expressing GFP–*PTEN* under low magnification were referenced to Hoechst/propidium iodide on the same field by switching fluorescent filters.

[^{3}H] Thymidine incorporation

Cells were seeded in 35-mm plates and infected with adenoviral wild-type *PTEN* or phosphatase-deficient *PTEN*. Forty-eight hours after infection, the cells were serum deprived for 48hr in DMEM plus 300 μg/ml G418 (GIBCO BRL). The cells were treated as indicated and cultured in 2 ml of fresh medium containing 1 μCi/ml [methyl-^{3}H] thymidine 5′-triphosphate (NEN Life Science Products), 300 μg/ml G418 for another 18 hr. Serum and hypoxia were administered simultaneously to the cultures. To eliminate the possibility that variations in cell density between plates would cause variations in ^{3}H incorporation, two additional dishes were plated and used to count cell number for each transfection at the time when [^{3}H] thymidine was added. For harvesting cells, the growth medium containing [^{3}H] thymidine was removed, and the cells were washed twice with PBS. The cells were rinsed twice with 2 ml of ice-cold 5% TCA and lysed by incubation in 1.5 ml of 0.25 M NaOH for 15 min at room temperature. A 0.6 ml-aliquot of each lysate was used for counting [^{3}H] thymidine incorporation. For all experiments, triplicate plates were used and mean values were graphed.

Immunoblots and kinase assays

Akt and phospho-Akt were immunoprecipitated, resolved by 12.5% SDS-PAGE, transferred to PVDF, blotted using PhosphoPlus Akt (Ser-473) Antibody Kit (New England Biolabs), visualized using a Vistra Western ECF Blotting Kit (Amersham L.S.), and quantitated by Fluorimager (Molecular Dynamics). In vitro kinase reactions were performed for 40 min at 30°C with constant shaking in 30-μl reaction volumes containing 500 ng of histone H2B, GST–jun, GST–GSK-3β, or immunoprecipitated HIF-1α as substrates, 10 μCi of [^{32}P] ATP, and immunoprecipitations of active Akt or JNK-1 as described (Datta et al. 1997). Kinase reactions were stopped by the addition of SDS-PAGE loading buffer and boiling for 5 min. The kinase reaction products were resolved by 12.5% SDS-PAGE, and the gels were washed for 5 min in dH_2O, dried, and visualized by PhosphorImaging (Molecular Dynamics).

Constructs, expression, and reporter assays

Construction and preparation of the VEGF and HRE luciferase reporter constructs, *p110**, *PLXN–PTEN*, *myr–Akt*, *myr–Akt–ER*, and *myr–A2–ER* were described previously (Hu et al. 1995; Mazure et al. 1997; Haas-Kogan et al. 1998; Kohn et al. 1998). Retroviral infection was performed by three sequential 6-hr incubations containing 5 μg/ml Polybrene, followed by recovery for 2–6 days. Adenoviruses expressing the *PTEN* or *PTENΔ* genes and capable of replicating in the "packaging" 293 cell line were made using the pAdEasy protocol. The virus was stored in single-use aliquots at –80°C. U373 cells were routinely infected at an MOI of 10 and cells were harvested 48 hr post infection. Transfections were performed using Lipofectamine Plus (GIBCO BRL). Luciferase assays were performed using the TB161 Luciferase Reporter Kit (Promega) and quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

RNA isolation and Northern blotting

Total RNA was isolated with TRIzol (GIBCO BRL). Total RNA/lane (10 μg) was denatured with glyoxal and size fractionated by electrophoresis on 1.4% agarose/sodium phosphate gels. RNA was transferred to nylon membranes and UV cross-linked. *PGK*, *PFK*, and *COX-1* were purchased as GEM clones (Genome Systems). Probes were cut out of the inserts with *Eco*RI and *Not*I from pINCY. The *VEGF* probe comprises a 600-bp fragment of the 5′UTR of *VEGF*. Radiolabeled probes were generated by random priming (Rediprime, Amersham) of cDNAs representing the complete coding sequences of *PGK*, *PFK*, c-*fos*, and *COX-1*. Blots were prehybridized and hybridized in ExpressHyb solution (Clontech) at 65°C, washed several times in 2× SSC/0.05% SDS and 0.2× SSC/0.1% SDS at 65°C, exposed to a PhosphorImager plate overnight, and visualized on a Storm 860 PhosphorImager (Molecular Dynamics).

Acknowledgments

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Tumor hypoxia and expression of c-met in cervical cancer

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Abstract

Objectives. Hypoxia enhances malignant progression by promoting the development of metastases and increasing invasiveness. One key regulator that controls growth, invasion and metastasis in cancer cells is the growth factor receptor c-met. The aim of this study, therefore, was to investigate the expression of the c-met protooncogene in cervical cancers in relation to intratumoral hypoxia levels and to clinico-pathological parameters.

Methods. 43 Patients with cervical cancer were subjected to intratumoral pO_2 measurement with the Eppendorf electrode and biopsies were taken. The tissue was subsequently analyzed by immunohistochemistry with an anti-c-met antibody.

Results. c-met was expressed in 72% of cervical cancers. There was a significantly stronger expression in poorly differentiated tumors ($r=0.4$, $p=0.008$). Furthermore, c-met expression was significantly associated with a spray-like pattern of invasion ($p=0.008$). However, there was no significant relationship between c-met expression and intratumoral hypoxia, pT stage, FIGO stage, lymphovascular space involvement, tumor size or overall survival.

Conclusions. Although c-met has been shown to be hypoxia-induced in vitro, our results suggest that it is not the mediator of deleterious effects of hypoxia on clinical outcome in cervical cancer.

Keywords: Tumor hypoxia; Tumor microenvironment; Gene regulation; Cervix; Uterus; Metastasis; Cancer

Introduction

Tumor hypoxia is a feature of many solid tumors including cervical cancer [1], head and neck cancer [2] and soft tissue sarcoma [3]. Clinical studies performed by our group showed that hypoxia is an independent prognostic indicator of poor outcome in patients with cervical cancer. Patients with hypoxic cervical cancers had a significantly worse prognosis compared to patients with better oxygenated tumors regardless of treatment modality [1]. Mechanisms by which hypoxia enhances malignant progression, thereby promoting the development of metastases and increasing invasiveness, comprise changes in gene expression [4,5] and clonal selection of cells that have lost their apoptotic potential [6,7].

One key regulator that controls growth, invasion and metastasis in cancer cells is the protein product of the proto-oncogene c-met. c-met encodes for a transmembrane tyrosine kinase acting as a growth factor receptor [8–10]. The ligand for this receptor is the hepatocyte growth factor (HGF, scatter factor-1) [11]. Inappropriate activation of the HGF pathway has been described as one major pathway in the process of invasive growth and the development of metastatic disease in malignant tumors [12–14]. Moreover, the c-met receptor has been shown to be overexpressed in a variety of human malignancies, including breast cancer, nasopharyngeal cancer, colon cancer and cervical cancer [15–18]. Using cell lines from different tumors including the cervical cancer cell line SiHa, Pennacchietti et al. [19] demonstrated that hypoxia activates the transcription of the met protooncogene, resulting

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in higher levels of the met protein. Furthermore, they showed that the met protein was highly overexpressed in hypoxic areas of the according experimental tumors. To the best of our knowledge, there are no studies to date addressing the relation between the expression of c-met in cervical cancer and intratumoral hypoxia levels. Therefore, we examined the expression of c-met in clinical samples of cervical cancer and investigated its relation to the hypoxia levels of the tumors determined by invasive measurements using the Eppendorf electrode. Furthermore, we analyzed the association between c-met expression and clinico-pathological parameters.

Material and methods

Patients, pO_2 measurement and tissue specimens

Archival tissue of cervical cancer samples with known intratumoral hypoxia levels was used for the analysis. The material was derived from a series of 43 consecutive patients for whom sufficient tumor material for analysis was available. These patients presented to the Department of Gynecology and Obstetrics at Leipzig University between 2001 and 2003 (FIGO stages IB to IV, Table 1) and prior to therapy underwent intratumoral oxygenation measurements with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier [20]. The patients were treated by Total Mesometrial Resection (TMMR [21]), Laterally Extended Endopelvic Resection (LEER [22]) or radiation therapy (Table 1). The procedure was performed after informed written consent was given by each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was performed in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. Per track, approximately 30 data points were collected, starting at a tissue depth of 5 mm. To confirm that the measurement was performed within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of approximately 2 mm in diameter and 20 mm in length was taken from the tissue of each measured track after the procedure. The biopsies were formalin-fixed and paraffin-embedded according to standard protocols, followed by an evaluation by a gynecologic pathologist. A correlation analysis between the median pO_2 of each track and the c-met protein expression in the corresponding biopsy was carried out (see below).

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures. 5-μm sections were stained for c-met with a rabbit polyclonal antibody (clone: c-12, Santa Cruz Biotechnology, Santa Cruz, USA, Cat-no: sc 10, dilution: 1:200). Briefly, slides were incubated overnight with the anti-c-met-antibody at 4 °C. This was followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO (DAKO Cytomation, Glostrup, Denmark). Staining was visualized by using DAB chromogen (DAKO). Sections known to stain positively as well as placental tissue were included in each batch as positive controls and negative controls were performed by omitting the primary antibody.

Evaluation of immunostaining

For the assessment of the c-met staining results, the slides were evaluated semiquantitatively using a predefined scoring system based on the product of staining intensity and percentage of positive tumor cells [23]. Membranous and/or cytoplasmic staining was counted as positive as described by Cruz et al. [24]. The staining of the slides was evaluated based on the microanatomic distribution of staining results using established criteria [25,26]. Briefly, staining intensity was evaluated as negative (0=no staining of cytoplasm or cell membrane), weak (1=fine fibrillar staining of cytoplasm and/or punctuate and incomplete membrane staining), moderate (2=granular/dotted staining of cytoplasm and/or weak to moderate staining of entire membrane), or strong (3=diffuse dark staining of cytoplasm and/or strong staining of entire membrane). The percentage of positive tumor cells was categorized as follows: 0=0%; 1=1–10%; 2=11–50%; 3=51–80%; 4>80%. By multiplying both components, an expression score (0–12) was obtained. This score was used in correlation analyses. Tissue specimens with a score of 0–2 were considered negative for c-met expression, a score of 4–6 represented moderate staining and a score of 9–12 was counted as strong staining.

Table 1
Patient and tumor characteristics at the time of pretherapeutic *p*O2 measurements

	No. of patients
FIGO stage	
I	11
II	17
III	11
IV	4
Grade	
1	9
2	26
3	8
pT stage	
pT1b1	11
pT1b2	3
pT2b	5
pT4	1
NA	23
pN stage	
N0	16
N1	4
NA	23
LVSI	
L0	19
L1	24
Tumor diameter (mm)	
(*n*=40, ND 3)	
Median	45
Range	17–100
Patient age (years)	
Median (range)	46 (24–79)
Treatment modality	
TMMR with pelvic±paraaortic lymph node dissection	19
LEER	1
Radiation therapy	23
Histology	
Squamous cell carcinoma	40
Adenocarcinoma	3

Abbreviations: FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; ND, not documented; LVSI, lymphovascular space involvement; TMMR, Total Mesometrial Resection; LEER, Laterally Extended Endopelvic Resection.

Statistical analysis

Correlations between two parameters were described by Spearman's rank correlation coefficient. The Mann–Whitney *U* test and Kruskal–Wallis *H* test were used for comparison of groups. Overall survival (OS), with deaths due to any cause as event, was calculated using the Kaplan–Meier method, and differences between groups were analyzed by log rank test.

A p value <0.05 was considered to indicate statistical significance. Statistical analysis was performed using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany).

Results

c-met expression in cervical cancer

c-met protein expression was assessed by immunohistochemistry in 43 samples. Negative staining was found in 28%, moderate staining in 40% and strong c-met staining in 32% of the investigated cervical cancers (Fig. 1). Tumor cells with membranous and/or cytoplasmic staining were considered positive.

c-met expression and intratumoral hypoxia

The intratumoral oxygenation of the investigated 43 cervical cancers had been determined using the Eppendorf electrode. The median pO_2 was 6.8 mm Hg (mean: 10.2 mm Hg, range: 0.8 mm Hg–33.4 mm Hg). There was no correlation between c-met staining of the core biopsies and the median pO_2 levels of the corresponding measured tracks (Spearman rank correlation, $r=0.19$, $p=0.22$, Fig. 2).

Tumor oxygenation, clinico-pathological parameters and survival

There was no significant relationship between tumor hypoxia and FIGO stage, tumor size, lymphovascular space involvement, lymph node status, histological grade or histological type, respectively (data not shown).

The median follow-up period was 42 months (95% CI: 39–45). For two patients, no follow-up data were available for survival analysis. There were no significant differences in overall survival comparing patients having well-oxygenated cervical cancers ($pO_2 > 10$ mm Hg) with patients having hypoxic tumors ($pO_2 \leq 10$ mm Hg) (3-year survival rate: 81.3% vs. 68.0%, $p=0.59$).

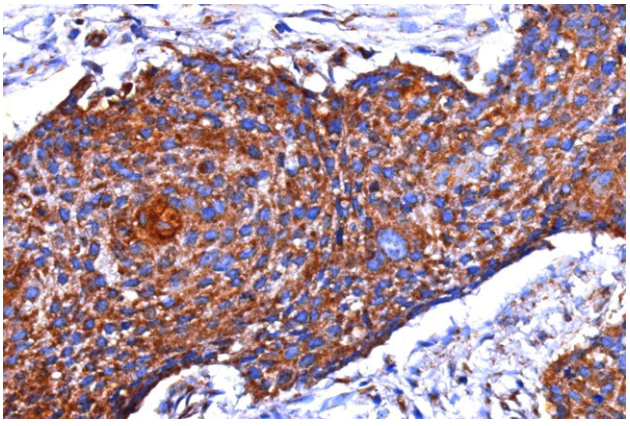

Fig. 1. Strong staining for c-met of tumor cells in a cervical cancer specimen (200× magnification).

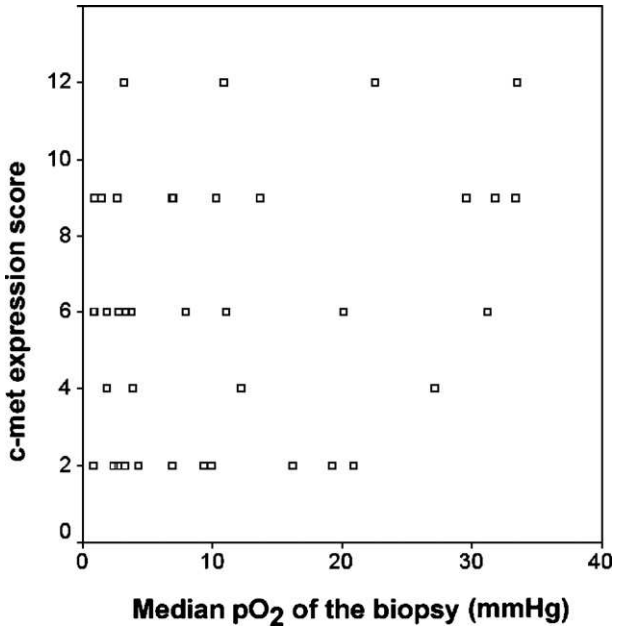

Fig. 2. Lack of correlation between the intratumoral median pO_2 levels and c-met expression in the corresponding biopsies.

c-met expression, clinico-pathological parameters and survival

c-met expression correlated significantly to histological tumor grade (Spearman rank correlation, $r=0.4$, $p=0.008$). This association was also evident in the box plot presentation (Kruskal–Wallis H test, $p=0.031$; Fig. 3). However, in the investigated cancers, there was no association between c-met expression and tumor size, lymph node status, lymphovascular space involvement, FIGO stage and histology (data not shown).

In the Kaplan–Meier analysis, there were no significant differences in overall survival comparing c-met-negative cervical

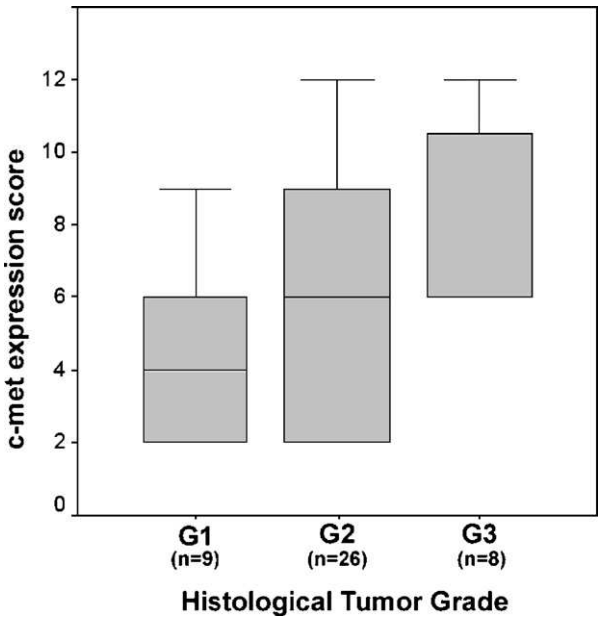

Fig. 3. Association between c-met expression in cervical cancers and histological tumor grade (G1, G2, G3).

cancers (score 0–2) with c-met-positive tumors (score>2) (3-year survival rate: 81.8% vs. 70.0%, $p=0.31$).

c-met expression and pattern of invasion

Of the 43 cervical cancers, 34 cases showed a finger-like pattern of invasion (Fig. 4A) and nine cancers exhibited a spray-like pattern (Fig. 4B). The degree of c-met expression was significantly associated with a spray-like pattern of invasion (Mann–Whitney U test, $p=0.008$, Fig. 4C).

Discussion

To our knowledge, this study explores for the first time the relation between c-met expression and intratumoral hypoxia levels in clinical samples of cervical cancer. We show that 72% of the investigated cancers displayed moderate or strong c-met staining. This is in line with a report by Baykal et al. who found c-met expression in 60% of the investigated cervical cancers [16]. A recent study by Tsai et al. [27] reported c-met expression in only 30% of their analyzed cancers, however, they exclusively studied adenocarcinomas of the uterine cervix. Likewise, we observed c-met expression in only one of the three adenocarcinomas present in our cohort.

In our study, c-met expression was significantly associated with a spray-like pattern of invasion that is related to a more aggressive phenotype resulting in poorer prognosis [28,29].

In keeping with previous results, the majority of investigated cervical cancers had a median pO_2 below 10 mm Hg, the commonly used threshold for hypoxic tumors [1]. In a previous study, we found that hypoxic cervical cancers were associated with a poorer survival when compared to well-oxygenated tumors [1]. Likewise, in the present study, patients with hypoxic tumors ($pO_2 \leq 10$ mm Hg) also had lower survival rates when compared to the other tumors, although this trend did not reach statistical significance.

In 2003, Pennacchietti et al. [19] demonstrated that hypoxia activates the transcription of the met protooncogene, resulting in higher levels of the met protein in vitro. They also showed that c-met was highly overexpressed in hypoxic areas of the investigated experimental tumors, as defined by expression of the hypoxia-inducible factor 1 (Hif-1). Although hypoxia is the strongest stimulus for Hif-1 expression, this transcription factor can also be induced by other factors, e.g., oncogenes [30]. In our study, we did not observe a correlation between intratumoral hypoxia levels and c-met expression. This is in line with a recent clinical study that did not show a correlation between Hif-1 expression and intratumoral hypoxia levels measured invasively in cervical cancer [31]. Although a variety of genes have been shown to be hypoxia-inducible in vitro, their association to intratumoral pO_2 levels in human cancer is less well defined [5]. Of the many hypoxia-induced markers identified in vitro, so far only the glucose transporter Glut-1 and carbonic anhydrase IX (CA IX) have been shown to correlate with the intratumoral oxygenation status in vivo [32,33]. These observations point towards a complex and finely tuned regulation of hypoxia-inducible genes in vivo. This regulation is only inadequately

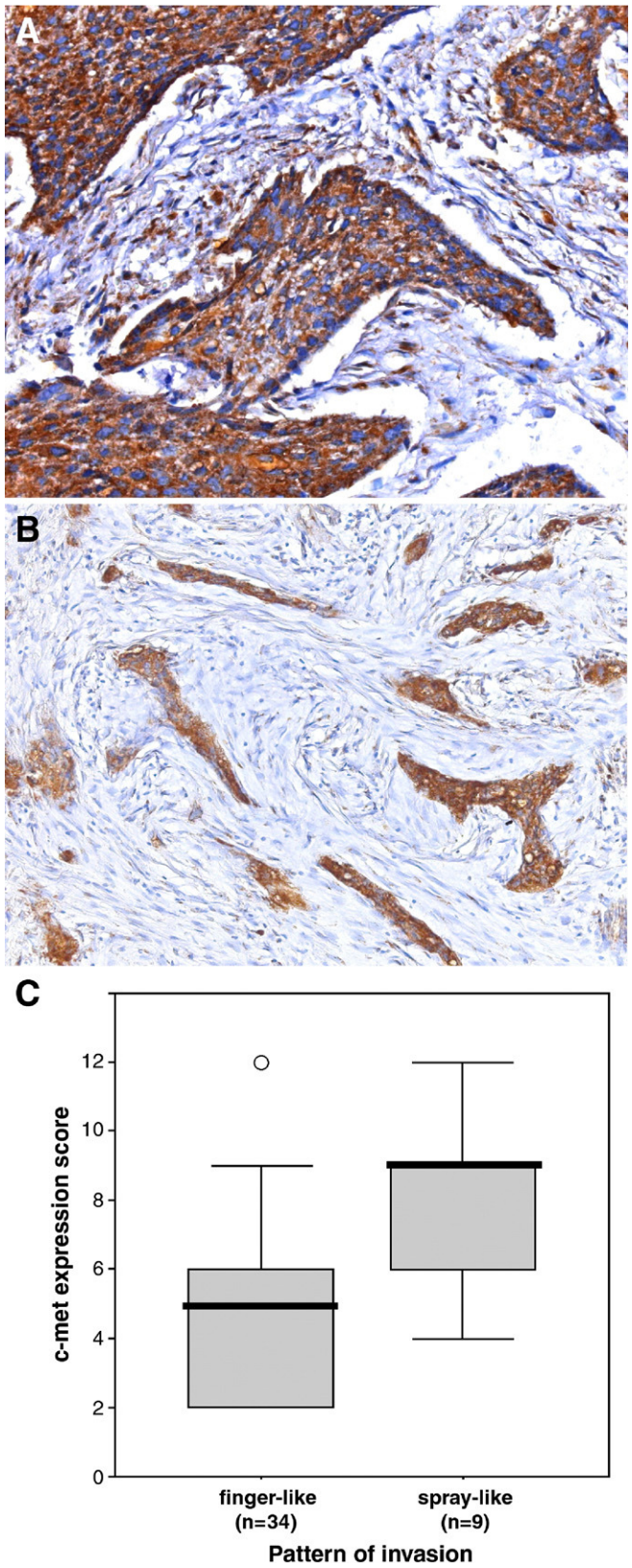

Fig. 4. (A) Cervical cancer exhibiting a finger-like pattern of invasion and strong c-met expression; (B) cervical cancer with a spray-like pattern of invasion showing strong c-met expression; (C) association between c-met expression and pattern of invasion.

represented in currently used in vitro models of hypoxia which typically consist in a one-time course of hypoxia lasting up to 48 h [34–36] neglecting the occurrence of chronic hypoxia and periods of reoxygenation.

Our results, therefore, suggest an additional or alternative regulation of c-met expression in vivo. Furthermore, it can be concluded that the deleterious effects of hypoxia on treatment outcome are likely independent of changes in c-met expression.

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Hypoxia and expression of the proapoptotic regulator BNIP3 in cervical cancer

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Abstract. Leo C, Horn L-C, Höckel M. Hypoxia and expression of the proapoptotic regulator BNIP3 in cervical cancer. *Int J Gynecol Cancer* 2006;**16:**1314–1320.

Hypoxia plays a major role in the malignant progression of tumors. Here, we investigate the expression of Bcl-2/adenovirus E1B 19 kd-interacting protein 3 (BNIP3), a proapoptotic Bcl-2 family member, and its relationship to hypoxia in cervical cancer cell lines and clinical samples of cervical cancer. Cervical cancer cell lines were grown under hypoxia or normoxia, and BNIP3 mRNA expression was examined by Northern blot analysis. In 50 patients with cervical cancer, intratumoral oxygen measurement with the Eppendorf electrode and needle biopsies of the tumor were performed. The obtained tissue was subsequently analyzed by immunohistochemistry with an anti-BNIP3 antibody. Cervical cancer tissue collected upon surgery was used for Northern blot analysis of *in vivo* BNIP3 mRNA expression. BNIP3 mRNA is strongly induced under hypoxic conditions in all cervical cancer cell lines investigated. Furthermore, Northern blot analysis revealed that BNIP3 mRNA is expressed in cervical cancer tissue. Using immunohistochemistry, we demonstrated that BNIP3 protein is expressed in 82% of the investigated cervical cancers and that more advanced tumor stages showed significantly stronger BNIP3 expression. However, we observed no correlation between BNIP3 expression and intratumoral hypoxia. In conclusion, BNIP3 is expressed in different cervical cancer cell lines as well as in clinical samples of cervical cancer. Although BNIP3 is clearly hypoxia-inducible *in vitro*, our results suggest additional mechanisms of BNIP3 regulation *in vivo*. Our findings therefore highlight a discrepancy between *in vitro* models of tumor hypoxia and the complexity of human cancer.

KEYWORDS: apoptosis, Bcl-2 family, gene regulation, tumor hypoxia, tumor microenvironment.

Hypoxia plays a major role in the malignant progression of tumors. Clinical studies performed by our group showed that patients with hypoxic cervical cancers had a significantly worse prognosis compared to patients with better oxygenated tumors, regardless of treatment modality[1]. Some of the differences between hypoxic and nonhypoxic tumor cells are mediated through differential regulation of gene expression and through the selection of tumor cells that resist hypoxia-induced apoptosis[2–4].

Bcl-2/adenovirus E1B 19 kd-interacting protein 3 (BNIP3) is a mitochondrial protein and a proapoptotic member of the Bcl-2 family[5]. Moreover, BNIP3 and its homolog NIX are expressed in different human tissues and strong expression was revealed in breast cancer compared to normal breast tissue[6]. BNIP3 and NIX were shown to be induced by hypoxia via the transcription factor Hif-1 in several cancer cell lines[6,7]. In addition, BNIP3 has been implicated in cardiac myocyte apoptosis[8], brain ischemia,[9] and chemotherapy resistance[10]. To our knowledge, the expression of BNIP3 in cervical cancer and its relation to hypoxia *in vivo* have not been analyzed to date.

In this study, we show BNIP3 to be highly induced by hypoxia in different cervical cancer cell lines. We also investigated the expression of BNIP3 mRNA in cervical cancer specimens. Using immunohistochemistry in an analysis of 50 cervical cancer samples, we found a significantly higher BNIP3 expression in tumors of more advanced clinical stages. However, we observed no correlation between intratumoral hypoxia and BNIP3 protein expression *in vivo*.

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Materials, patients, and methods

Cell culture and hypoxic conditions

Cervical cancer cell lines SiHa, Caski, and C33a were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For hypoxic conditions, cells were plated in plastic dishes and placed in a hypoxia chamber (Invivo$_2$ 400; Ruskinn Technology Ltd., UK) under 0.1% oxygen conditions at a confluency of 70–80%. After defined time points, cells were removed from the chamber and total RNA was isolated.

RNA isolation and Northern blotting

Tissue was prepared with an Ultra-Turrax T8 homogenizer (IKA, Stauffen, Germany). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Fifteen micrograms of total RNA per lane was size-fractionated on 1% agarose gels containing formaldehyde. RNA was transferred to nylon membranes (Schleicher & Schuell, Dassel, Germany) and UV cross-linked. Radiolabeled probes of cDNAs representing the BNIP3 complete coding sequence were generated by random priming (Rediprime; Amersham Biosciences, Freiburg, Germany). L28 cDNA was used as a loading control. Blots were prehybridized and hybridized in ExpressHyb solution (BD Biosciences Clontech, Palo Alto, CA) at 62°C, washed several times in 2× SSC/0.05% SDS and 0.2× SSC/0.1% SDS at 56°C, exposed to a phosphorimager plate overnight, and visualized on a Bio-Rad Phosphorimager (Bio-Rad, Hercules, CA). All experiments were performed in triplicate.

Patients, pO_2 measurement, and tissue specimens

Fifty patients with cervical cancer (FIGO stage IB to IV; Table 1) presenting to the Department of Gynecology and Obstetrics at Leipzig University between January 2001 and January 2003 underwent intratumoral oxygenation measurement with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier[11]. The procedure was performed after informed written consent was given by each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was performed pretherapeutically in the conscious patient. For each patient, pO_2 measurement within the macroscopically vital tumor generated approximately 30 oxygenation data points per track starting at a tissue depth of 5 mm. To confirm that the measurement was performed within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of approximately 2 mm in diameter and 20 mm in length was taken of each measured track after the procedure. The biopsies were formalin-fixed and paraffin-embedded according to standard protocols, followed by an evaluation by a gynecological pathologist. A correlation analysis between the median pO_2 of each track and BNIP3 protein expression in the corresponding biopsy was performed (see below). For Northern blot analysis, cervical cancer tissue as well as tumor-surrounding tissue from seven patients who underwent surgical therapy was shock-frozen in liquid nitrogen and stored at −80°C.

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures. Five-micrometer sections were stained with a rabbit polyclonal anti-BNIP3 antibody (Cat.-Nr.: 12-01-16401; Biocarta Europe GmbH, Hamburg, Germany). Briefly, slides were boiled in Target retrieval solution (DAKO Cytomation, Glostrup, Denmark) for 20 min in a pressure cooker for antigen demasking and then incubated with the anti-BNIP3 antibody (dilution 1:1000) overnight at 4°C, followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO. Staining was visualized by using DAB chromogen (DAKO). Negative controls were performed by omitting the anti-BNIP3 antibody in the primary antibody incubation.

Evaluation of BNIP3 immunostaining

BNIP3 staining intensity (negative/weak, moderate, strong) and staining quantity (percentage of stained tumor cells) of each pO_2-measured biopsy were evaluated semiquantitatively by two independent investigators (L.C.H., C.L.) as described previously[12]. The investigators were blinded to the patient data and oxygenation measurements. In cases with discrepant assessments, an agreement was obtained after collegial revision. In accordance to Giatromanolaki *et al.*[13], cytoplasmic as well as nuclear staining results were counted as positive.

Statistical analysis

Statistical analysis was performed using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany). Correlations between two parameters were described by Spearman's rank correlation

Table 1. Patient and tumor characteristics at the time of pretherapeutic pO_2 measurements

	No. of patients	Median	Range
FIGO stage			
I	10		
II	16		
III	19		
IV	5		
Grade			
1	6		
2	33		
3	11		
pT stage			
pT1b1	13		
pT1b2	2		
pT2b	3		
pT4	1		
NA	31		
pN stage			
N0	16		
N1	3		
NA	31		
LVSI			
L0	16		
L1	32		
ND	2		
Tumor diameter (mm)	48	45	15–100
ND	2		
Patient age (years)			
≤50	36	47	24–79
>50	14		
Treatment modality			
Radical hysterectomy with pelvic ± para-aortic lymph node dissection	18		
Primary exenteration	1		
Radiation therapy	31		
Tumor oxygenation pO_2 (mm Hg)			
≤10 mm Hg	29	7.4	0–33.3
>10 mm Hg	21		

FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; ND, not documented; NA, not applicable because treated by radiation therapy; LVSI, lymphovascular space involvement.

coefficient (rho). Fisher's exact test was used for comparison of categorized variables. A P value ≤0.05 was considered to indicate statistical significance.

Results

BNIP3 mRNA expression in cervical cancer cell lines

BNIP3 mRNA is induced strongly under hypoxic conditions in all three cervical cancer cell lines investigated (Fig. 1). All experiments were performed in triplicate and statistical analysis was performed using the mean values of the three independent experiments. There is only a low basal BNIP3 level in all cell lines and the maximum of induction is reached after 12 h of culture under 0.1% oxygen. We quantified the level of BNIP3 mRNA induction in these cell lines and found a 14- to 17-fold increase over baseline at the 12-h time point.

Expression of BNIP3 mRNA and protein in cervical cancers

Northern blot analysis showed a marked BNIP3 mRNA expression in five of the seven investigated cervical cancers (Fig. 2).

Immunohistochemistry was performed in 50 cervical cancers and BNIP3 protein expression was observed in 82% of the investigated cases. Specifically, weak BNIP3 staining was found in 32%, moderate staining in 30%, and strong staining intensity in 20% of all cases (Fig. 3). Positive tumor cells presented a diffuse, sometimes granular cytoplasmic staining. Some tumors showed an additional nuclear staining. Some peritumoral mononuclear inflammatory cells displayed cytoplasmic staining also.

BNIP3 expression and intratumoral pO_2

For the 50 tumors, the median oxygenation along the histologically confirmed single tracks used for BNIP3 immunostaining was 7.4 mm Hg (mean pO_2 11.3 mm Hg, range 0–33.3 mm Hg). Statistical analysis revealed no correlation of tumor oxygenation with the number of BNIP3-expressing cells or with staining intensity (Fig. 4a, b). Notably, we found a group of seven hypoxic cancers (median pO_2 ≤10 mm Hg) with absent BNIP3 protein expression (see discussion). However, statistical analysis did not reveal differences between clinicopathologic parameters in this group compared to all other carcinomas.

BNIP3 expression and clinicopathologic parameters

Tumors with more advanced clinical FIGO stages showed a significantly stronger BNIP3 staining intensity ($P = 0.028$, Table 2). We observed no correlation between BNIP3 expression and histological grade, lymphovascular space involvement, tumor diameter, pathological tumor stage, lymph node status, histology type, or the presence of necrosis ($P > 0.05$, Table 2).

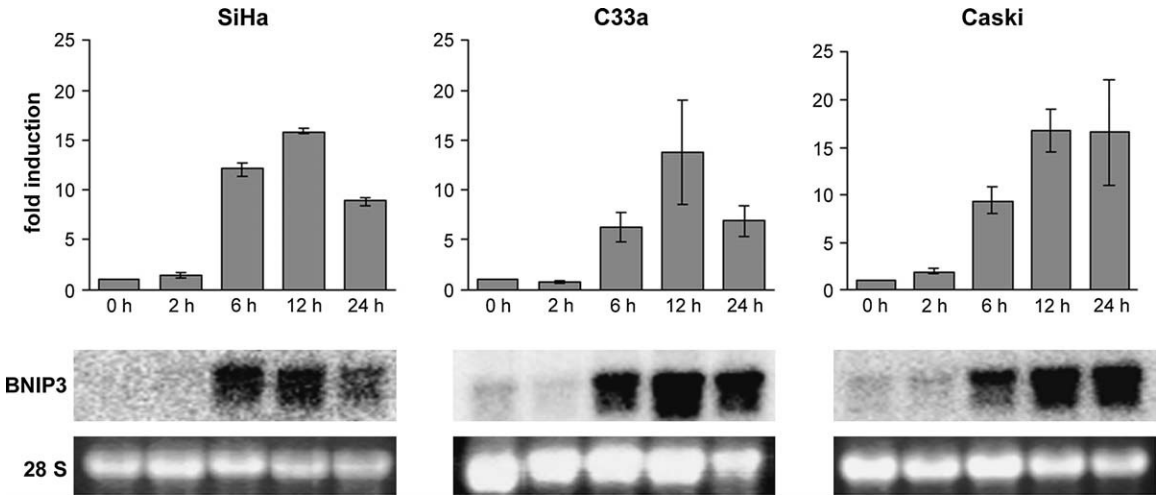

Figure 1. Northern blot analysis of BNIP3 expression in three cervical cancer cell lines (SiHA, C33a, and Caski). The diagrams in the top panels show the induction of BNIP3 mRNA at defined time points of hypoxia treatment. All experiments were performed in triplicate. The bottom panels show representative Northern blots, the ethidium bromide–stained ribosomal 28S band serves as a loading control.

Tumor oxygenation, tumor staging, and clinicopathologic parameters

There was no significant correlation between tumor hypoxia and FIGO stage ($P = 0.11$, data not shown). The group of surgically treated patients for which a pathologic tumor stage is available consisted of 19 patients and, therefore, was too small for statistical evaluation. There was no correlation between intratumoral pO_2 and tumor size, lymph node status, lymphovascular space involvement, and histological grade ($P > 0.05$; data not shown).

Discussion

To our knowledge, this study demonstrates for the first time the expression of BNIP3 mRNA and protein in cervical cancer cell lines and clinical samples of cervical cancer. We show that BNIP3 mRNA is induced by hypoxia in three cervical cancer cell lines and is expressed in cervical cancer specimens. These findings are consistent with the results of a study regarding the expression of BNIP3 in breast cancer[6], which reported upregulation of the mRNA in cancerous but not in normal breast tissue. In the same study, the authors observed an upregulation of BNIP3 mRNA and protein in several cancer cell lines and found this induction to be regulated by the transcription factor Hif-1. Another study by the same group showed a significant correlation between BNIP3 expression in ductal carcinoma *in situ* of the breast and the histologic grade of the lesion as well as the occurrence of comedoid tumor necroses[14].

This is the first report that investigates the dependency of BNIP3 expression on intratumoral pO_2 *in vivo*. As needle core biopsies were collected directly following oxygenation measurement, we were able to perform the immunohistochemical analysis on tissue samples with a defined pO_2 value. This approach allows a more precise description of the spatial relationship between intratumoral oxygenation and BNIP3 expression than methods that correlate the expression in a tissue section to the overall pO_2 of the tumor. In keeping with previous results, the majority of investigated cervical cancers had a median pO_2 below 10 mm Hg, the commonly used threshold for hypoxic tumors[1]. Although our *in vitro* study demonstrates a significant induction of BNIP3 mRNA by hypoxia in all investigated cervical cancer cell lines, our *in vivo* data from cervical cancer patients fail to show a correlation between BNIP3 protein expression and intratumoral pO_2. While we did not analyze BNIP3 protein expression in cervical cancer cell lines, Sowter et al. previously demonstrated a congruity between

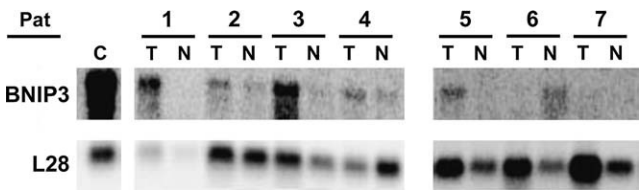

Figure 2. Northern blot analysis of BNIP3 expression in cervical cancers (T) and corresponding tumor-free surrounding cervical tissue (N). Control (C): mRNA of the cervical cancer cell line Caski at 12 h of hypoxia. The mRNA of the ribosomal protein L28 serves as a loading control.

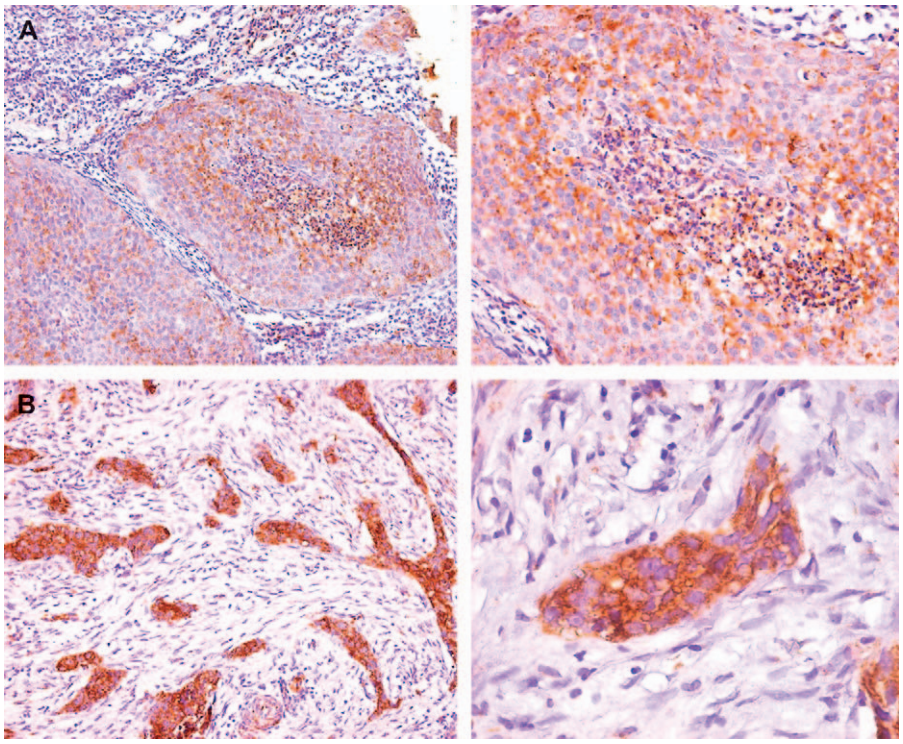

Figure 3. Immunohistochemical analysis of BNIP3 protein expression in two different cervical cancer specimens. A) weak expression, B) strong expression (×200 and ×400 magnification).

hypoxia-induced mRNA and protein expression in a variety of other cancer cell lines[6]. Therefore, our results suggest that hypoxia-independent mechanisms may contribute to BNIP3 regulation in human cancers. In accordance with Giatromanolaki *et al.*[13], our investigation did not show a relationship between BNIP3 expression and the presence of necrosis.

Although a variety of genes have been shown to be hypoxia-inducible *in vitro*, the association between intratumoral pO_2 and the expression of endogenous hypoxia markers in human cancer is less well defined[15]. Of the many hypoxia-induced markers identified *in vitro*, so far only the glucose transporter Glut-1 and carbonic anhydrase IX (CA IX) have been shown to correlate with the intratumoral oxygenation status *in vivo*. Glut-1 expression was increased in regions of intratumoral hypoxia measured both by pimonidazol binding and Eppendorf needle electrode in cervical cancer[16,17]. Moreover, absence of Glut-1 was associated with metastasis-free survival[16]. Similarly, CA IX expression correlated positively to the level of intratumoral hypoxia in cervical cancer and was associated with poor survival in cervical, breast, and lung cancer in some studies[18–20]. However, a recent study by Hedley *et al.*[21] showed no correlation of CA IX expression with intratumoral hypoxia and outcome in patients with cervical cancer. Additionally, neither Hif-1 nor the proangiogenic factor VEGF, known to be

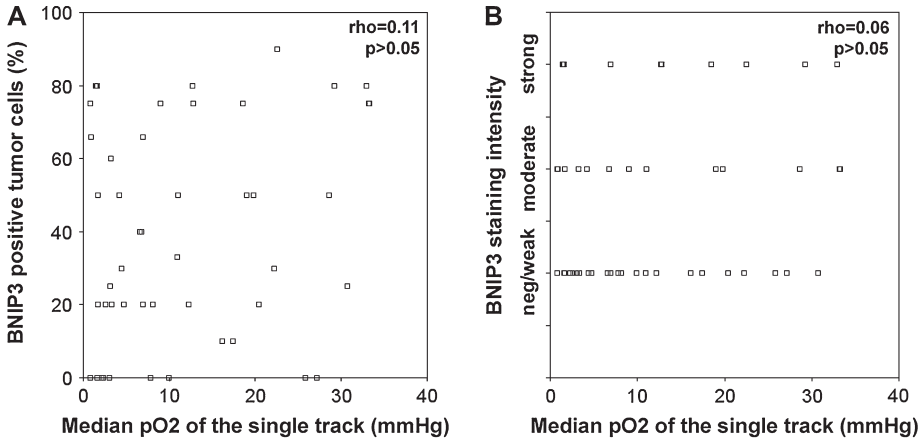

Figure 4. A) Percentage of BNIP3-expressing tumor cells as a function of the median pO_2 values of the corresponding core biopsies of cervical cancer specimens. Each data point represents one measured track. B) BNIP3 staining intensity as a function of the median pO_2 of the cervical cancer biopsies (BNIP3 staining intensity categorized into three groups: negative to weak, moderate, and strong).

Table 2. BNIP3 association with clinical and histologic parameters

		BNIP3			
		Negative/weak	Moderate	Strong	*P* value
Clinical parameters					
Stage	FIGO I	7	3	0	$P = 0.028$
	FIGO II	10	4	2	
	FIGO III	4	7	8	
	FIGO IV	4	1	0	
Histologic parameters					
pT	pT1b1	10	3	0	$P > 0.05$
	pT1b2	2	0	0	
	pT2b	2	1	0	
	pT4	1	0	0	
pN	pN0	13	3	0	$P > 0.05$
	pN1	2	1	0	
LVSI	L0	6	8	2	$P > 0.05$
	L1	19	7	6	
Grade	G1	5	1	0	$P > 0.05$
	G2	14	13	6	
	G3	6	1	4	
Tumor diameter*	≤45 mm	15	8	2	$P > 0.05$
	>45 mm	10	6	7	
Histology type	SCC	20	14	10	$P > 0.05$
	AC	4	1	0	
	ASC	1	0	0	
Necrosis	No	15	11	8	$P > 0.05$
	Yes	10	4	2	

FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; LVSI, lymphovascular space involvement; *, for two carcinomas the diameter was not documented; SCC, squamous cell cancer; AC, adenocarcinoma; ASC, adenosquamous carcinoma.

hypoxia-inducible *in vitro*, showed an association with intratumoral oxygenation measured with the Eppendorf electrode in two clinical studies[22,23]. Likewise, the association between metallothionein expression and hypoxia observed *in vitro* was not found in human patients with squamous cell cancer of the head and neck or the uterine cervix[24].

These observations point toward a complex and finely tuned regulation of these hypoxia-inducible genes *in vivo* that warrants further elucidation. In cancer, hypoxia results from the increased oxygen demand of the growing tumor, which at some point exceeds its blood supply. In order to adapt to this condition, hypoxic tissues upregulate several angiogenic factors[25]. The resulting tumor vasculature displays a pathologic and chaotic architecture maintaining the hypoxic micromilieu and, thus, hypoxic gene activation[26,27]. On the other hand, sprouting vessels, thrombosis, and fibrinolysis are assumed to lead to periods of transient reperfusion with reoxygenation[28]. This complexity of human tumors is only inadequately represented in currently used *in vitro* models of hypoxia, which typically consist of a one-time course of hypoxia lasting up to 48 h[2,29,30].

In the present study, we showed that tumors with a more advanced FIGO stage had significantly stronger BNIP3 expression. Whether this is an effect of advanced tumor progression or whether BNIP3 could serve as a prognostic marker in cervical cancer warrants further elucidation. Giatromanolaki *et al.*[13] found the proapoptotic BNIP3 to be a prognostic marker of poor survival in non-small cell lung cancer. This unexpected finding could be explained by the lack of posttranslational mechanisms activating BNIP3. Furthermore, BNIP3 is induced by Hif-1[6] and could be a marker of increased Hif-1 signaling rather than being a prognostic marker in itself. Finally, the role of hypoxia in apoptosis is ambiguous. On the one hand, hypoxia is a stimulus for apoptosis[31,32]. On the other hand, hypoxia selects for apoptosis-resistant cells *in vitro* and *in vivo*[3]. Building on these experimental data, a study in cervical cancer patients by Höckel *et al.*[4] revealed a group of hypoxic cancers characterized by a low apoptotic index, indicating that they had grown resistant to apoptosis. These cervical cancers had a significantly worse prognosis than hypoxic tumors with a higher apoptotic index. While the present study did not find a correlation of BNIP3

expression with the tumor pO_2, we identified a group of hypoxic cervical cancers with a lack of expression of the proapoptotic BNIP3. Future studies will have to address whether the loss of BNIP3 expression in certain hypoxic tumors leads to apoptosis resistance and affects clinical outcome in cervical cancer patients.

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Expression of Erythropoietin and Erythropoietin Receptor in Cervical Cancer and Relationship to Survival, Hypoxia, and Apoptosis

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Abstract

Purpose: Physiologically, hypoxia induces the expression of erythropoietin (Epo) in adult kidney cells. Epo, in turn, acts on the Epo receptor (EpoR) in RBC precursors to stimulate growth and prevent apoptosis. Because hypoxia plays a major role in the malignant progression of tumors and Epo and its receptors have also been detected in malignant tumors, we investigated the expression of Epo and EpoR and their relationship with hypoxia, proliferation, apoptosis, and clinicopathologic variables in cervical cancer.

Experimental Design: Intratumoral oxygen measurement and needle biopsies of the tumors were done in 48 patients with cervical cancer. The obtained tissue was analyzed by immunohistochemistry with antibodies against Epo, EpoR, and Ki-67 as well as by terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling assays.

Results: Epo and EpoR were expressed in 88% and 92% of samples, respectively. Cervical cancers with higher Epo expression showed a significantly reduced overall survival (3 years, 50.0% versus 80.6%; $P = 0.0084$). Epo and EpoR expression correlated significantly with apoptosis ($r = 0.49$, $P = 0.001$ and $r = 0.36$, $P = 0.021$). Furthermore, EpoR expression correlated significantly with tumor size ($r = 0.32$, $P = 0.032$) and was significantly associated with the presence of lymphovascular space involvement ($P = 0.037$). However, we observed no correlation between Epo or EpoR expression and intratumoral hypoxia, although in well-oxygenated tumors, EpoR localized significantly more often to the invasion front ($P = 0.047$).

Conclusions: This study analyzes Epo/EpoR expression and their relationship with intratumoral pO_2 levels as well as with survival in patients with cervical cancer. The data suggest a critical role of the endogenous Epo/EpoR system in cervical cancer.

Hypoxia plays a major role in the malignant progression of solid tumors. Hypoxic microregions have been detected in a wide variety of solid tumors (1–3). Previous clinical studies done by our group showed that patients with hypoxic cervical cancers had a significantly worse prognosis compared with patients with better oxygenated tumors regardless of treatment modality. Tumor hypoxia was shown to be the most powerful independent prognostic factor in cervical cancer (1). Mechanisms by which sustained tumor hypoxia may increase aggressiveness include differential regulation of gene expression (4) and clonal selection of tumor cells that have lost their apoptotic potential (5, 6). One of the transcription factors most sensitive to a hypoxic micromilieu is the hypoxia-inducible factor 1 (Hif-1; ref. 7). Induction of Hif-1 results in the upregulation of a wide variety of target genes including genes involved in metabolism, angiogenesis, metastasis/invasion, as well as apoptosis (8). A major Hif-1 target gene is erythropoietin (Epo; ref. 9). Epo receptor (EpoR) is also induced by hypoxia but is not regulated by Hif-1 (10, 11).

Epo is a glycoprotein hormone stimulator of erythropoiesis (12) produced in the kidneys and liver, and exerts its effect by stimulating growth, preventing apoptosis, and inducing differentiation of RBC precursors (13). EpoR belongs to the cytokine receptor superfamily (14). Recent studies have detected the expression of Epo and EpoR not only in normal nonhematopoietic tissues and cells, including the central nervous system and vascular endothelial cells (15, 16), but also in a variety of solid tumors (10, 11, 17–22).

Recombinant human Epo is used to treat chemotherapy-induced anemia. Although two large clinical trials documented negative effects of recombinant human Epo on patient outcome in head and neck as well as breast cancer (23, 24), a recent meta-analysis did not find an unfavorable effect on overall survival of the treated cancer patients (25).

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Recently, EpoR was shown *in vitro* to be up-regulated in cancer cell lines under hypoxic conditions (10, 11). However, the relationship between intratumoral pO_2 and the expression of hypoxia-related proteins in human cancer is still not clear. Thus far, the existence of a clear spatial association between hypoxia and the expression of Hif-1 or its targets in clinical cancer samples remains controversial (26–30).

This study investigates the expression of Epo and EpoR and their correlation with intratumoral pO_2 levels in cervical cancer. Furthermore, we studied the association between Epo/EpoR expression and apoptosis, proliferation, and clinicopathologic variables of the respective tumors.

Materials and Methods

Patients, pO_2 measurement, and tissue specimens. Archival tissue of cervical cancer samples with known intratumoral hypoxia levels was used for the analysis. The material was derived from a series of 48 consecutive patients for whom sufficient tumor material for analysis was available. These patients presented to the Department of Gynecology and Obstetrics at Leipzig University between 2001 and 2003 [International Federation of Gynecologists and Obstetricians (FIGO) stage IB-IV; Table 1] and prior to therapy underwent intratumoral oxygenation measurements with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier (31). The procedure was done after informed written consent was given by each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was done in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. For each track, approximately 30 data points were collected starting at a tissue depth of 5 mm. To confirm that the measurement was done within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of ~2 mm in diameter and 20 mm in length was taken of each measured track after the procedure. The biopsies were formalin-fixed and paraffin-embedded according to standard protocols, followed by an evaluation by a gynecologic pathologist. A correlation analysis between the median pO_2 of each track and Epo and EpoR protein expression in the corresponding biopsy was done (see below).

Immunohistochemical staining for Epo, EpoR, and Ki-67. Immunohistochemical staining was done according to standard procedures. The sections were stained with rabbit polyclonal anti-Epo and anti-EpoR antibodies (Epo: H-162, sc-7956; EpoR: C-20, sc-695; both from Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal anti-Ki-67 antibody (clone Ki-S5; DAKO, Carpinteria, CA).

Briefly, after blocking of endogenous peroxidase and tissue avidin and biotin (DAKO), slides were incubated with the anti-Epo antibody (dilution, 1:100) overnight at 4°C, followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO. Staining was visualized by using 3,3′-diaminobenzidine chromogen (DAKO). For EpoR, slides were incubated with the anti-EpoR antibody (dilution, 1:250) overnight at 4°C, followed by incubation with a biotinylated Pan-specific antibody (horse biotinylated anti-mouse/rabbit/goat IgG; Vector Laboratories, Burlingame, CA) and the Vectastain Elite ABC system (Vector Laboratories). Staining was visualized by using 3,3′-diaminobenzidine chromogen. Negative controls were done by omitting the respective antibodies in the primary antibody incubation. For Epo and EpoR, slides of adult kidney (32) and placenta (33) were used as positive controls.

For Ki-67, slides were boiled in target retrieval solution (DAKO) for 30 minutes in a pressure cooker for antigen demasking, and incubated overnight with the anti-Ki-67 antibody (dilution, 1:50) at 4°C. This was followed by incubation with a biotinylated secondary antibody (link anti-mouse antibody, DAKO) and the CSA system from DAKO. Staining was visualized by using 3,3′-diaminobenzidine chromogen.

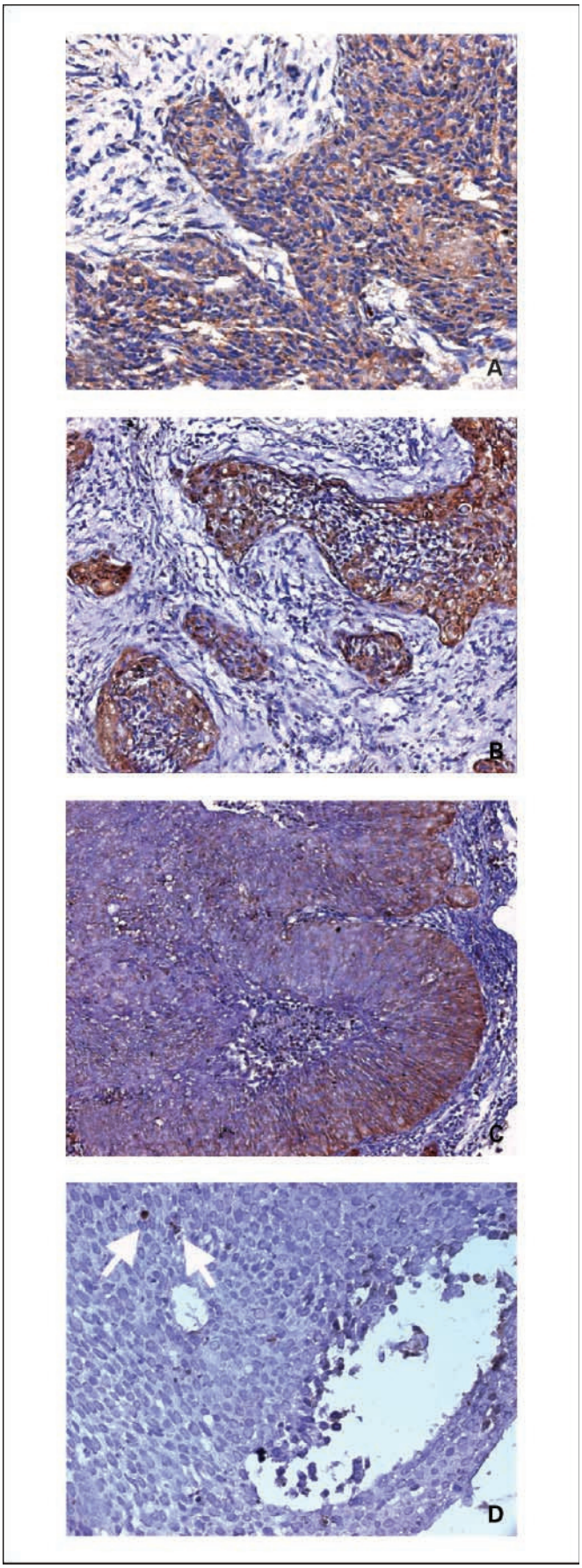
Table 1. Patient and tumor characteristics at the time of pretherapeutic pO_2 measurements

	No. of patients
FIGO stage	
I	12
II	17
III	13
IV	6
Grade	
1	8
2	27
3	13
pT stage	
pT_{1b1}	15
pT_{1b2}	2
pT_{2b}	2
pT_4	1
n.a.	28
pN stage	
N_0	16
N_1	4
n.a.	28
Lymphovascular space involvement	
L0	15
L1	33
Tumor diameter (mm)	
Median (range)	44 (17-100)
Patient age (y)	
Median (range)	47 (24-79)
Treatment modality	
Radical hysterectomy with pelvic ± paraaortic lymph node dissection	19
Primary exenteration	1
Radiation therapy	28
Tumor oxygenation pO_2 (mm Hg)	
Median (range)	8.6 (0.8-33.3)
≤10 mm Hg	26
>10 mm Hg	22

Abbreviations: pT stage, pathologic tumor stage; pN stage, pathologic node stage; n.a., not applicable (treated by radiation therapy).

Terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling assays. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuracil triphosphate (dUTP) nick-end labeling (TUNEL). Slides were treated with the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the instructions of the manufacturer. Briefly, after routine deparaffinization, sections were digested with proteinase K (20 mg/mL) for 5 minutes at 37°C and incubated with the reaction mixture (1:100) for 60 minutes at 37°C. This was followed by incubation with a streptavidin-peroxidase complex (1:500) for 30 minutes at room temperature and subsequent color development with 3,3′-diaminobenzidine. As positive controls, DNase-treated lymph node sections were used, and for negative controls, the terminal deoxynucleotidyl transferase enzyme was omitted.

Evaluation of immunostaining. For the evaluation of cytoplasmic staining results for Epo and EpoR, a predefined scoring system based on the product of staining intensity and the percentage of positive tumor cells was used (21). Staining intensity was evaluated as negative (0), weak (1), moderate (2), strong (3), and the percentage of positive tumor cells was categorized as follows: (0) 0%, (1) 1% to 10%, (2) 11% to 50%, (3) 51% to 80%, and (4) > 80%. By multiplying both components, an expression score (0-12) was obtained. This score was



used for the correlation analyses. Evaluation of the samples was done by two independent investigators who were blinded to the patient data. In cases of discrepant assessment, an agreement was obtained after collegial revision using a multiheaded microscope.

To assess the effect of Epo and EpoR on survival, the Epo and EpoR expression scores were divided into high and low scores using the median expression score for Epo and EpoR, respectively, as the cutpoint (21).

Cells with clear brown nuclear labeling were defined as Ki-67- or TUNEL–positive, respectively. For the Ki-67 labeling index, 1,000 tumor cells were counted under 400× magnification, and the rate of Ki-67 positive cells was calculated as a percentage. Analogously, to determine the apoptotic index (AI) of a tumor, the number of terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling–positive cells in 1,000 tumor cells was expressed as a percentage.

Statistical analysis. To evaluate the association between ordinal data, the Spearman correlation coefficient was calculated, and for categorical data, χ^2 test was used. Groups were compared by use of Kruskal-Wallis *H* test and Mann-Whitney *U* test. Overall survival, with deaths due to any cause as event, and relapse-free survival, with relapse and metastases as events, were analyzed by log rank test. Kaplan-Meier curves and 3-year survival rates with 95% confidence intervals (95% CI) are presented. Cox regression analysis was done to assess the effect of Epo on overall survival as adjusted for FIGO stage and treatment modality. FIGO stage was dichotomized into early (FIGO I-II) and advanced (FIGO III-IV). The estimator of the effect is expressed as relative risk with 95% CI and corresponding *P* values.

$P < 0.05$ were considered to indicate statistical significance. Statistical analysis was done using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany).

Results

Patient characteristics and clinicopathologic features. All cervical carcinomas were clinically staged according to FIGO criteria. The median age at diagnosis was 47 years (range, 24-79 years). In 19 of the examined cases, the tumor was resected by total mesometrial resection (34) along with pelvic/para-aortic lymph node dissection. In one case (FIGO IV), the tumor was treated with curative intent by primary laterally extended endopelvic exenteration (35). For these patients, the tumors were additionally staged according to the pTNM system. Twenty-eight patients were treated by radiation therapy. The distribution of FIGO and TNM stages is shown in Table 1. Forty-one tumors were of squamous cell origin, six cancers represented adenocarcinomas, and one was an adenosquamous cell carcinoma. The median hemoglobin level at the time of biopsy was 8.1 mmol/L (range, 5.6-9.6 mmol/L). For two patients, the hemoglobin levels were not available.

Expression of Epo and EpoR protein in cervical cancers. Immunohistochemistry was done in all 48 cervical cancer samples. Epo protein expression was observed in 88% of the investigated cases. Positive tumor cells presented a diffuse, cytoplasmic staining (Fig. 1A). In 25% of cancers, Epo expression was accentuated at

Fig. 1. *A,* diffuse cytoplasmic staining for Epo in squamous cell carcinoma of the uterine cervix (original magnification, ×214). *B,* strong cytoplasmic staining for EpoR in squamous cell carcinoma of the uterine cervix (original magnification, ×214). *C,* pronounced staining for EpoR at the front of invasion in squamous cell carcinoma of the uterine cervix (original magnification, ×150). *D,* dark stained cells, terminal deoxynucleotidyl transferase – mediated deoxyuracil triphosphate nick-end labeling – positive cells in cervical cancer (*arrows*).

the infiltrating edge of the respective tumor. Proximity to necrosis did not influence the Epo expression pattern (data not shown).

For EpoR, cytoplasmic immunostaining was present in 92% of cervical cancers (Fig. 1B). In 38% of all samples, EpoR expression was more pronounced at the infiltrating edge of tumors (Fig. 1C). Adjacent necroses did not have an influence on EpoR expression (data not shown). There was a significant positive correlation between Epo and EpoR protein expression in the investigated tumors (r = 0.36, P = 0.013) and in 81% of the cancers, coexpression of Epo and EpoR was found.

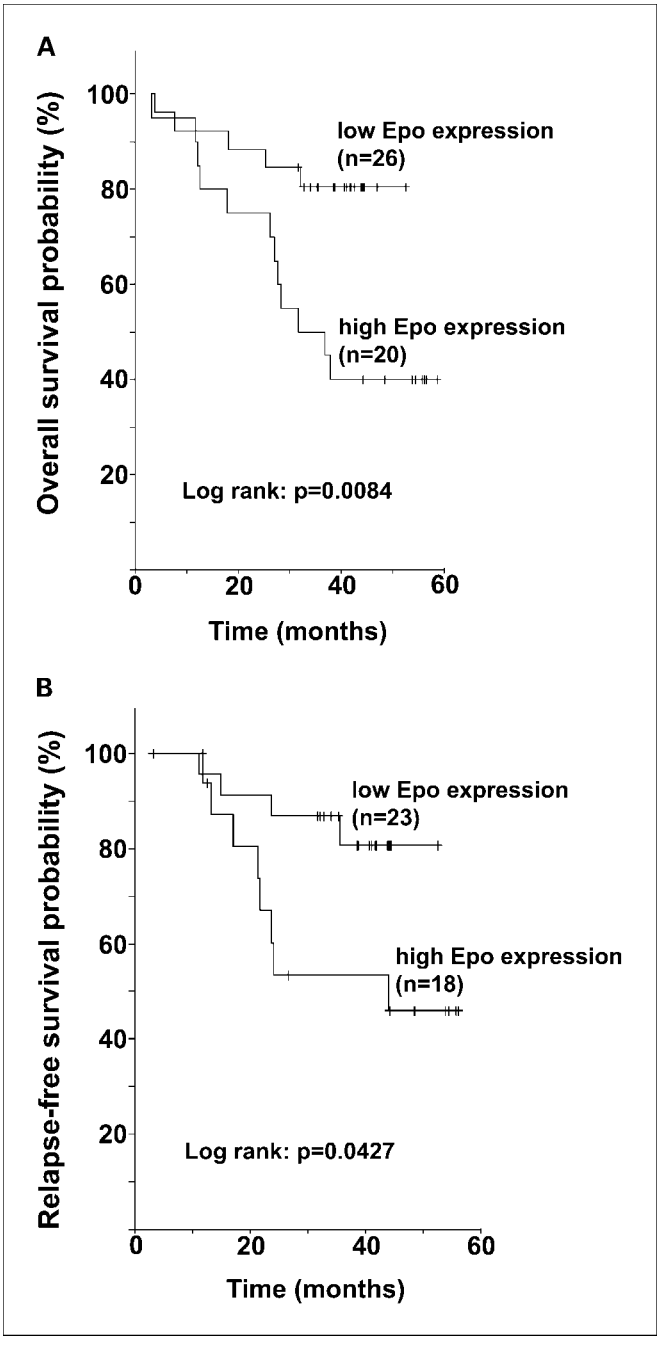


Fig. 2. Kaplan-Meier analysis showing that high Epo expression significantly correlates with reduced overall survival (*A*) and reduced relapse-free survival (*B*) in patients with cervical cancer.

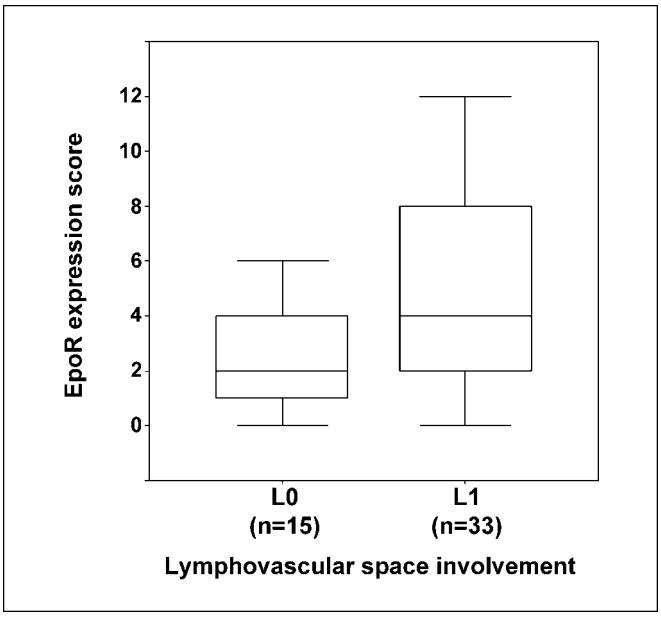


Fig. 3. EpoR expression in cervical cancers without (L0) and with (L1) lymphovascular space involvement.

Epo and EpoR expression and intratumoral pO_2. For the 48 tumors, the median oxygenation along the histologically confirmed single tracks used for Epo and EpoR immunostaining was 8.9 mm Hg (range, 0.8-33.3 mm Hg). There was no correlation between the intratumoral oxygenation and Epo or EpoR expression, respectively (Epo: $r = -0.08$, $P = 0.59$; EpoR: $r = 0.24$, $P = 0.096$). In tumors with a median pO_2 >10 mm Hg, the EpoR expression localized significantly more often with the infiltrating edge of the tumor (57% versus 27%, $P = 0.047$), whereas no such association could be found for Epo.

Epo and EpoR expression and clinical outcome. The median follow-up period was 44 months (95% CI, 41.4-46.8). For two patients, no follow-up data were available for survival analysis. Five patients (four with disease progression and one case with unknown relapse status) were not included in the analysis for relapse-free survival. As described in Materials and Methods, the median expression score for Epo (median = 2) and EpoR (median = 2) was used to compare low-expressing and high-expressing groups for survival. To analyze the effect of Epo on overall survival, univariate and multivariate Cox regression models were calculated. Patients with a high Epo expression score had a significantly reduced overall survival [3-year rate: 50.0% (95% CI, 28.1-71.9%) versus 80.6% (95% CI, 65.3-95.9%); $P = 0.008$; Fig. 2A] and a significantly reduced relapse-free survival [3-year rate: 53.6% (95% CI, 28.3-78.8%) versus 80.8% (95% CI, 63.4-98.1%); $P = 0.043$; Fig. 2B]. Epo had an independent significant effect on overall survival after adjustment for FIGO stage and treatment modality (relative risk, 3.0; 95% CI, 1.0-8.8%; $P = 0.047$). Hemoglobin levels had no relevant clinical effect in univariate or multivariate analyses, nor did they influence the observed effect of Epo on survival.

For EpoR, there was no significant difference in overall survival and relapse-free survival comparing the high EpoR expression and low EpoR expression groups (overall survival,

3-year rate: 56.5% versus 78.2%, $P = 0.136$; relapse-free survival, 3-year rate: 68.8% versus 70.3%; $P = 0.719$).

Epo and EpoR expression and clinicopathologic variables. Tumors with lymphovascular space involvement showed significantly higher EpoR scores ($P = 0.037$; Fig. 3). Furthermore, there was a significant positive correlation between EpoR expression and tumor size ($r = 0.32$, $P = 0.032$). We observed no correlation between Epo expression and lymphovascular space involvement or tumor diameter. Also, there was no association between Epo/EpoR expression and histologic grade, FIGO stage, lymph node status, histology type, or hemoglobin level (Table 2).

Epo and EpoR expression, proliferation, and apoptosis. The AI and Ki-67 labeling index were determined in 40 of the 48 cervical carcinomas. The remaining eight cases were excluded because no sufficient material from the needle core biopsies was left for analyses. The median AI was found to be 1.2% (range, 0.3-3.4%; Fig. 1D), whereas the median Ki-67 labeling index was 36.6% (range, 8.5-75.8%). There was a significant positive correlation between Epo expression and AI ($r = 0.49$, $P = 0.001$l Fig. 4A) and between EpoR expression and AI ($r = 0.36$, $P = 0.021$; Fig. 4B). We found no correlation between Epo or EpoR expression and the Ki-67 labeling index ($r = -0.13$; $P = 0.43$; $r = -0.14$, $P = 0.39$).

Discussion

To our knowledge, this is the first study analyzing the effect of Epo and EpoR expression on the survival of patients with cervical cancer. Furthermore, the relationship of Epo and EpoR expression to intratumoral pO_2 levels and apoptosis in cervical cancer was investigated.

In our study, we found Epo expression in 88% and EpoR expression in 92% of cases. This is in line with findings by Acs et al. who showed Epo expression in 14 out of 15 cervical cancers, and showed EpoR expression in all investigated samples (11). In our cohort, cervical cancers with high Epo expression resulted in a significantly reduced overall and relapse-free survival, whereas no statistically significant survival difference was observed for high EpoR expression. This finding implies that Epo-expressing cervical cancers are more aggressive.

Recently, Epo and its receptor were shown in a variety of other solid tumors including head and neck cancer (19, 21), breast cancer (10, 36), non–small cell lung cancer (20), and endometrial cancer (17). Hypoxia is a feature of many solid tumors and may render a malignant tumor more aggressive (37). Although a great number of genes have been shown to be hypoxia-inducible *in vitro*, there is a paucity of studies investigating hypoxia-induced gene and protein expression in tumors with quantified pO_2 levels. Therefore, the association between intratumoral hypoxia and the expression of hypoxia-related markers is still not well defined (8). In our study, we compared Epo and EpoR expression to the degree of intratumoral hypoxia of the respective cervical cancers. To minimize the potential for sampling errors, needle core biopsies were collected directly following invasive oxygenation measurement. With this method, we were able to

Table 2. Association between clinicopathologic variables and Epo/EpoR expression

Clinicopathologic variables	Epo score median (min-max)	*P*	EpoR score median (min-max)	*P*
Histologic grade				
1	1 (0-6)	0.130*	1.5 (0-9)	0.134*
2	4 (0-12)		4 (0-12)	
3	2 (0-12)		2 (1-6)	
FIGO stage				
I	2 (0-6)	0.511*	1.5 (0-6)	0.063*
II	2 (0-12)		6 (0-12)	
III	2 (0-12)		2 (0-12)	
IV	2.5 (0-6)		4 (1-9)	
pN stage				
pN_0	2 (0-6)	0.601†	2 (0-9)	0.900†
pN_1	1 (0-9)		2 (2-2)	
Lymphovascular space involvement				
L0	2 (1-12)	0.897†	2 (0-6)	0.037†
L1	2 (0-12)		4 (0-12)	
Histology				
Squamous cell carcinoma	2 (0-12)	0.993*	2 (0-12)	0.222
Adenocarcinoma + adenosquamous cell carcinoma	2 (1-9)		2 (0-6)	

Clinicopathologic variables	Epo correlation coefficient (Spearman)	*P*	EpoR correlation coefficient (Spearman)	*P*
Hemoglobin level	0.034	0.832‡	0.071	0.639‡
AI	0.486	0.001‡	0.363	0.021‡
Ki-67 index	−0.129	0.427‡	−0.139	0.391‡
Tumor size	0.137	0.370‡	0.32	0.032‡

* Kruskal-Wallis *H* test was used to analyze the association.
† Mann-Whitney *U* test was used to analyze the association.
‡ Spearman rank correlation was used to analyze the association.

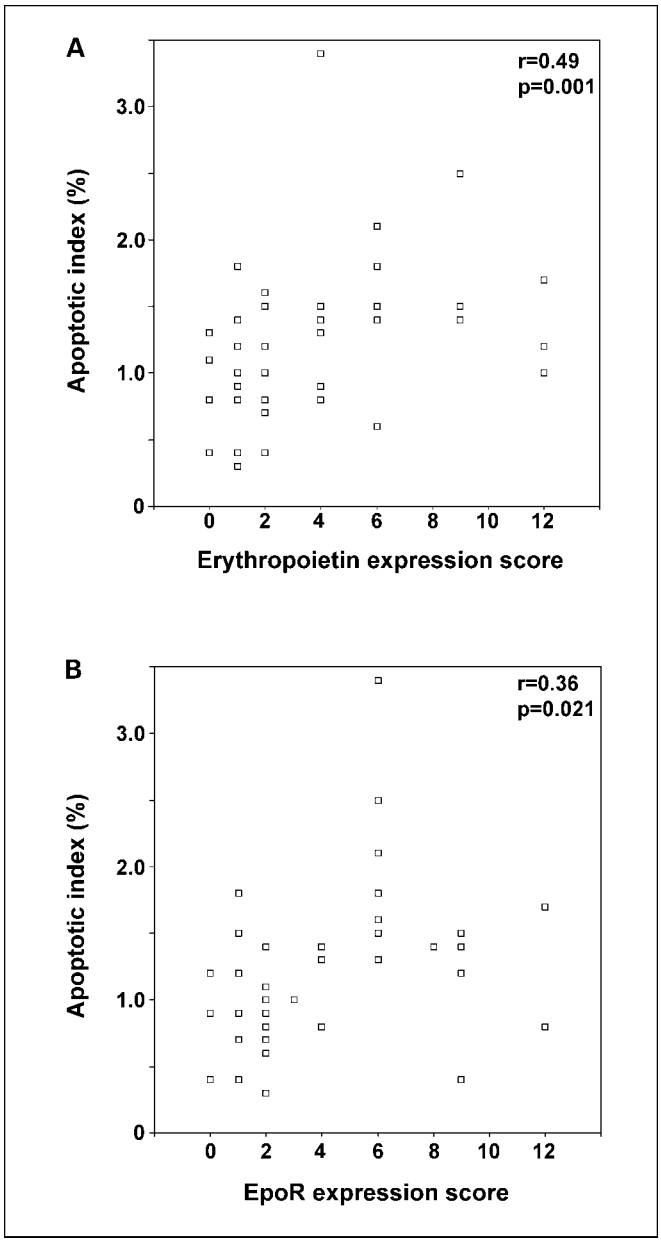


Fig. 4. *A,* positive correlation of immunohistochemical expression of Epo with the AI in cervical cancers. *B,* positive correlation of immunohistochemical expression of EpoR with the AI in cervical cancers.

perform the immunohistochemical analysis on tissue samples with a defined pO_2 value, allowing a precise description of the spatial relationship between intratumoral oxygenation and the respective protein expression. In our study, there was no significant correlation between EpoR expression and intratumoral oxygenation values, suggesting hypoxia-independent mechanisms of EpoR induction *in vivo*. However, in well-oxygenated cervical cancers with a median pO_2 of >10 mm Hg (the commonly used threshold for hypoxic cervical cancers; ref. 1), EpoR expression localized significantly more often to the infiltrating edge of tumors, an area thought to belong to the most hypoxic parts of solid cancers (38). This finding is therefore in agreement with the previously described hypoxia-inducible EpoR signaling in cervical cancer cell lines *in vitro* (11). In our samples, we did not observe a correlation between Epo expression and tumor hypoxia. Physiologically, Epo is up-regulated by Hif-1, the most sensitive and specific transcription factor under hypoxic conditions (39). A recent study by Winter et al. in head and neck squamous cell carcinoma showed a significant correlation between Epo and Hif-1 expression (21). The observed lack of a direct correlation between Epo expression and tumor hypoxia in our samples might be explained by the fact that Hif-1 is not only induced by hypoxia, but also by a variety of other stimuli, including tumor suppressor inactivation and oncogene activation (40). Furthermore, our observation is consistent with another clinical study in cervical cancer that showed no association between Hif-1 and intratumoral oxygenation measured with the Eppendorf electrode (30). Additionally, a recent report by Arcasoy et al. investigated Epo expression and tumor hypoxia determined by pimonidazole binding in head and neck squamous cell cancer (19). The authors showed that Epo expression did not always colocalize with regional tumor hypoxia as determined by pimonidazole binding. Another previous study in breast cancer also did not show consistent colocalization of Epo expression and hypoxia as determined by pimonidazole binding (36). These data suggest additional mechanisms of Epo induction in tumor cells.

In our cohort of cervical cancers, we found a significant positive correlation between the AI and Epo/EpoR expression, respectively. Our findings have several possible interpretations: first, Epo/EpoR pathways may not be functional in the investigated cervical cancers or may have a biological role that differs from their antiapoptotic and proliferative effects in hematopoiesis (13). Second, even in the presence of functional pathways, the Epo/EpoR system may fail to prevent apoptosis, e.g., because of alterations in sequence, structure, secretion, or subcellular localization of its components. Although evidence for an autocrine-paracrine influence of endogenous Epo on tumor cells has previously been reported (36, 41, 42), a recent *in vivo* study found no influence of recombinant human Epo on tumor growth, proliferation rate, and tumor angiogenesis (43). Third, the Epo/EpoR system might be up-regulated to compensate for the high apoptosis rates observed in a subset of our investigated cervical cancers and, subsequently, mediate antiapoptotic effects. This hypothesis is consistent with the observed reduced survival of patients with high Epo-expressing cervical cancers in our study. Additionally, this hypothesis could explain the negative effects of recombinant human Epo on patient outcome in head and neck as well as breast cancers (23, 24) that were documented in two large clinical trials, as exogenous Epo might further propagate malignant progression. Because other reports as well as a recent meta-analysis did not find an unfavorable effect on overall survival of the treated cancer patients (25), future functional studies will have to further characterize the role of the Epo/EpoR system in malignant tumors.

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Expression of Apaf-1 in cervical cancer correlates with lymph node metastasis but not with intratumoral hypoxia

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Abstract

Objectives. The aim of this study was to investigate the expression of the proapoptotic protein Apaf-1 in cervical cancers. Moreover, we studied its correlation to intratumoral pO_2 and to clinico-pathological parameters.

Methods. 86 patients with cervical cancer were subjected to intratumoral pO_2 measurement with the Eppendorf electrode. From these patients, cervical cancer tissue was used for immunohistochemistry with an anti-Apaf-1 antibody.

Results. Apaf-1 is expressed in cervical cancer. Cervical cancers with strong or moderate Apaf-1 expression had significantly less lymph node metastases at time of surgery than tumors with weak or negative Apaf-1 expression ($P = 0.022$). There was no significant correlation between Apaf-1 expression and intratumoral pO_2, pT stage, FIGO stage, lymphovascular space involvement, and grade.

Conclusions. Loss of Apaf-1 expression may represent a marker of aggressive tumor behavior since it correlates significantly with the occurrence of lymph node metastasis in cervical cancer.

Keywords: Tumor hypoxia; Metastasis; Cervical cancer

Introduction

Hypoxia plays an important role in the malignant progression of tumors. Clinical studies performed by our group showed that patients with hypoxic cervical cancers had a significantly worse prognosis compared to patients with better oxygenated tumors regardless of treatment modality [1]. Moreover, we showed that hypoxic cervical cancers with a low fraction of apoptotic cells are highly aggressive [2]. The mechanisms by which hypoxia confers this apoptosis resistance have only partially been elucidated. Graeber and co-workers [3] showed in a mouse model that hypoxic conditions select for apoptosis-resistant p53−/− mouse embryonic fibroblasts (MEFs). Furthermore, the cell death factors BNIP3 and NIX were shown to be induced under hypoxic conditions [4].

One critical regulator that plays a role in apoptosis under hypoxia is the apoptotic protease activating factor-1 (Apaf-1). Apaf-1 is a crucial part of the apoptosome that is assembled in response to several cellular stresses (i.e., hypoxia, DNA damage, oncogene activation, etc). Activation by these signals finally leads to caspase activation via the intrinsic mitochondrial pathway resulting in apoptotic cell death [5–7]. Apaf-1 knockout mice showed severe defects in the apoptotic response to hypoxic stimulation [8]. This finding demonstrates Apaf-1 as an essential component of the apoptotic response to hypoxia in vitro.

To our knowledge, the expression of Apaf-1 in cervical cancer has not been analyzed to date. Here we show that Apaf-1 is expressed in cervical cancer and that an absent or weak Apaf-1 expression correlates significantly with the presence of lymph node metastasis at time of surgery. In this

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study, we also investigated the intratumoral pO_2 in 86 cervical cancers and we show that there is no direct correlation between intratumoral hypoxia and Apaf-1 protein expression in vivo.

Material, patients, and methods

Patients, pO_2 measurement, and tissue specimens

86 patients with cervical cancer presenting to the Department of Gynecology at Leipzig University and Mainz University between 1993 and 2003 (FIGO stage IB to IV, Table 1) underwent intratumoral oxygenation measurements with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier [9]. The procedure was performed after informed written consent was given by each patient. The study was approved by the medical ethics committees of the respective universities. pO_2 measurement was performed pretherapeutically in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. Per track, approximately 30 data points were collected, starting at a tissue depth of 5 mm. The median pO_2 of all measured points was used to represent the oxygenation status of the respective tumor. From all patients, representative cervical cancer tissue was collected following oxygenation measurement or upon surgical treatment. The tissue was formalin-fixed, paraffin-embedded, and subjected to immunohistochemistry.

Table 1
Patient and tumor characteristics at the time of pretherapeutic pO_2 measurements

	No. of patients	Median	Range
FIGO stage			
I	20		
II	37		
III	22		
IV	7		
Grade			
1	11		
2	51		
3	24		
pT stage			
pT1b1	19		
pT1b2	3		
pT2b	23		
pT3b	2		
pT4	1		
NA	38		
pN stage			
N0	27		
N1	19		
NA	38		
ND	2		
LVSI			
L0	33		
L1	53		
Tumor diameter (mm) (n = 82, ND = 4)		45	17–100
Tumor oxygenation pO_2 (mm Hg) (n = 86)			
≤10	62	4.6	1.3–56.8
>10	24		

Abbreviations: FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; ND, not documented; NA, not applicable because treated by radiation therapy; LVSI, lymphovascular space involvement.

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures. 5-μm sections were stained with a polyclonal rabbit anti-Apaf-1 antibody (Cat. No. 2013, ProSci, Poway, USA). Briefly, slides were boiled in Target retrieval solution (DAKO Cytomation, Glostrup, Denmark) for 20 min in a pressure cooker for antigen demasking and incubated overnight with the anti-Apaf-1 antibody (dilution 1:200) at 4°C. This was followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO. Staining was visualized by using DAB chromogen (DAKO). Negative controls were performed by omitting the anti-Apaf-1 antibody in the primary antibody incubation.

Evaluation of Apaf-1 immunostaining

Apaf-1 staining intensity (negative/weak, moderate, strong) [10] and staining quantity (percentage of stained tumor cells) were evaluated semiquantitatively by two independent investigators (LCH, AS) who were blinded to the patient data and oxygenation measurements. In cases with discrepant assessments, an agreement was obtained after collegial revision. A section was only counted as negative when an internal control (endothelial or peritumoral inflammatory cells) was positive for Apaf-1.

Statistical analysis

Statistical analysis was performed using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany). Correlations between two parameters were described by Spearman's rank correlation coefficient (rho). Fisher's exact test, the Chi square test, or the Mann–Whitney test was used for comparison of categorized variables. A P value < 0.05 was considered to indicate statistical significance.

Results

Patient characteristics and clinico-pathological features

All cervical carcinomas were clinically staged according to FIGO criteria. The median age at diagnosis was 47 years

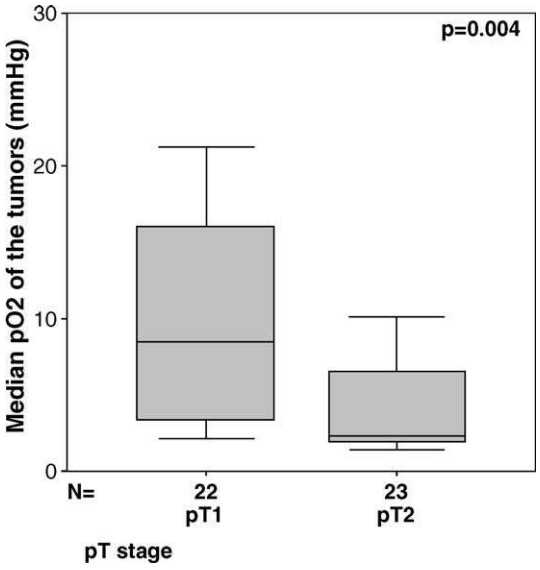

Fig. 1. The median pO_2 of each cervical cancer was measured with the Eppendorf electrode. Tumors with higher pathological tumor stages (pT2) had a significantly lower tumor oxygenation as described by the median pO_2.

(range 24–79 years). Of all the 86 examined cases, 38 patients received radiotherapy, while in 45 patients, the tumor was resected by either classical radical hysterectomy or total mesometrial resection along with pelvic/paraaortic lymph node dissection (TMMR) [11]. In the remaining three patients (two FIGO IIIb cases, one FIGO IV case), the tumors were treated under curative aspects by primary exenteration. The surgically manageable cases were classified according to TNM criteria [12]. The distribution of FIGO and TNM stages is shown in Table 1. Seventy-three tumors were of squamous cell origin, eight cancers were adenocarcinomas, and five were represented by adenosquamous cell carcinomas. In the TNM classified group, 27 patients were lymph node negative and 19 patients had lymph node metastases (in two cases, the nodal status was not documented).

Tumor oxygenation, tumor staging, and clinico-pathological parameters

The tumor oxygenation of 86 tumors was measured by the Eppendorf electrode. The median oxygenation was 4.6 mm Hg (mean: 8.2 mm Hg, range: 1.3–56.8 mm Hg). There was no significant correlation between tumor hypoxia and FIGO stage ($P = 0.11$, data not shown). However, an advanced histological pT stage (pathological tumor stage) correlated significantly with low intratumoral oxygenation in the group of patients treated with radical hysterectomy or TMMR ($P = 0.004$, Fig. 1). The group of FIGO III/IV cases contained only three surgically treated patients and, therefore, was too small for statistical evaluation. There was no correlation between intratumoral pO_2 and tumor size, lymph node status, lymphovascular space involvement, and histological grade, respectively ($P > 0.05$; data not shown).

Apaf-1 protein expression in cervical cancers

Apaf-1 protein expression was assessed by immunohistochemistry. Negative or weak Apaf-1 staining was found in 24%, moderate staining in 34%, and strong staining in 42% of all investigated cervical cancers. Positive tumor cells presented a diffuse, sometimes granular cytoplasmic staining (Fig. 2). Some peritumoral mononuclear inflammatory and stromal cells also displayed cytoplasmic staining.

Apaf-1 expression and intratumoral pO_2

Statistical analysis revealed no correlation of tumor oxygenation with the number of Apaf-1 expressing cells or with staining intensity (Fig. 3).

Apaf-1 and clinico-pathological parameters

Analyzing the surgically treated cases ($n = 46$; in two cases the nodal status was unavailable), we found a

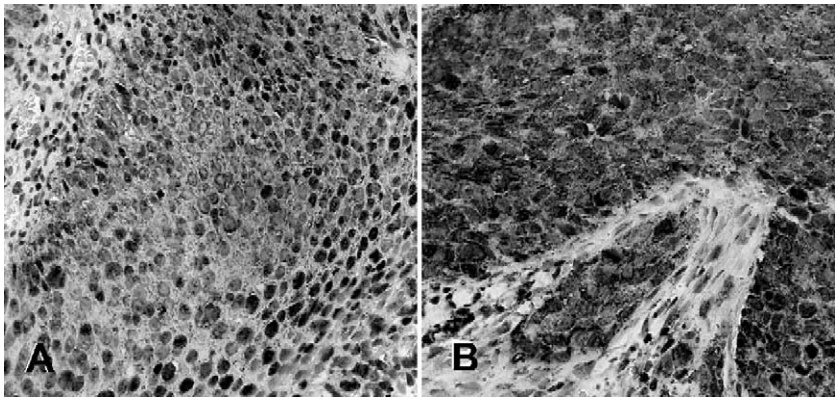

Fig. 2. Immunohistochemical analysis of Apaf-1 protein expression in two different cervical cancer specimens. A: weak expression, B: strong expression (200× magnification).

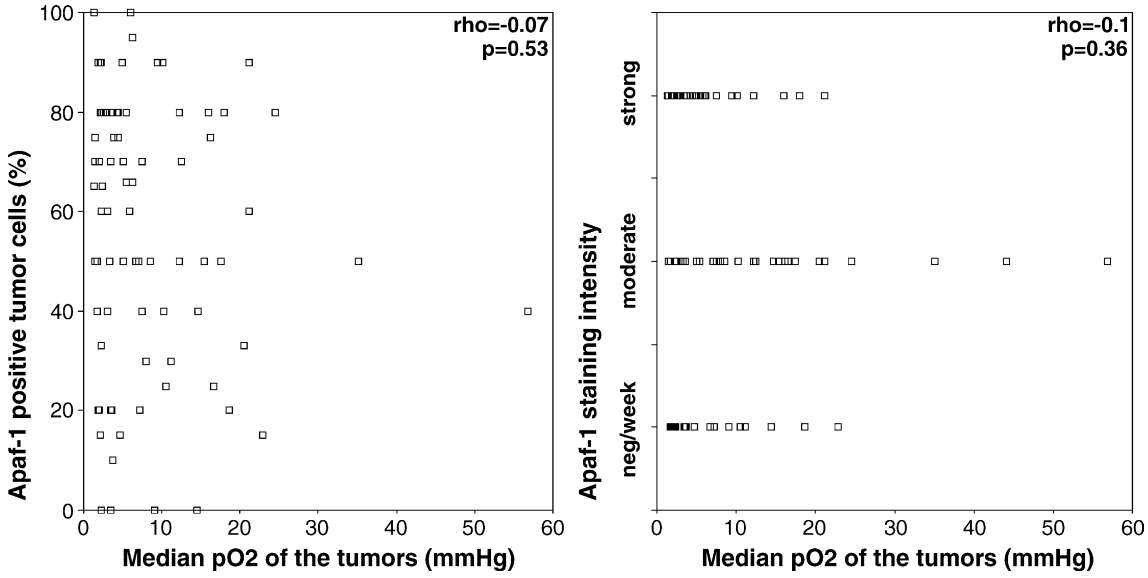

Fig. 3. Apaf-1 expressing tumor cells and Apaf-1 staining intensity as a function of the median pO_2 of the corresponding cervical cancers. There is no correlation between tumor oxygenation and Apaf-1 expression.

significant correlation between Apaf-1 expression and nodal status. Cervical cancers with strong or moderate Apaf-1 expression had significantly less lymph node metastases at time of surgery than tumors with weak or negative Apaf-1 expression (P = 0.022, Table 2). There was no significant correlation between Apaf-1 expression and pT stage, FIGO stage, lymphovascular space involvement, or grade (Table 2).

Table 2
Apaf-1 association with clinical and histological parameters

		Apaf-1			P value
		Neg/weak	Moderate	Strong	
Clinical parameters					
Stage, n = 86	FIGO I	5	8	7	
	FIGO II	13	13	11	
	FIGO III	1	6	15	
	FIGO IV	2	2	3	>0.05
Histological parameters					
pT, n = 48	pT1b1	4	9	6	
	pT1b2	0	0	3	
	pT2b	10	5	8	
	pT3b	1	1	0	
	pT4	0	1	0	>0.05
pN, n = 46[a]	pN0	4	12	11	
	pN1	10	4	5	0.022
LVSI, n = 86	L0	5	14	14	
	L1	16	15	22	>0.05
Grade, n = 86	G1	2	5	4	
	G2	12	17	22	
	G3	7	7	10	>0.05

Abbreviations: FIGO, International Federation of Gynecologists and Obstetricians; pT stage, pathologic tumor stage; pN stage, pathologic node stage; LVSI, lymphovascular space involvement.
[a] For two carcinomas, the pN status was not documented.

Discussion

To our knowledge, this study demonstrates for the first time the expression of the proapoptotic regulator Apaf-1 in clinical samples of cervical cancer. Furthermore, in this report, the dependency of Apaf-1 expression on intratumoral pO_2 in vivo and the relation to clinico-pathological parameters were examined. In keeping with previous results, the majority of investigated cervical cancers had a median pO_2 below 10 mm Hg, the commonly used threshold for hypoxic tumors [1]. In our study, tumors with more advanced pathological tumor stages were significantly more hypoxic. This finding is also consistent with our previous work where borderline significance between tumor oxygenation and pT stage was demonstrated [1].

Presumably, hypoxia occurs early in tumor development, implying an early selection pressure on cancer cells to escape hypoxia-induced apoptosis [13]. The resulting resistance to apoptosis may contribute to the aggressive phenotype that is characteristic for many hypoxic tumors. While Apaf-1 has been shown to be involved in activation of apoptosis in response to hypoxia in vitro [8], we did not find a direct correlation between Apaf-1 protein expression and intratumoral pO_2.

In the past years, several studies have demonstrated abolished Apaf-1 function in cancer. In pancreatic ductal adenocarcinoma, Kimura et al. found that the region containing the Apaf-1 locus was deleted in 60% of tested tumors [14]. Another study by Soengas et al. [15] demonstrated epigenetic silencing of Apaf-1 in malignant metastatic melanoma. Apaf-1-negative melanomas were invariably chemoresistant and unable to execute an apoptotic response following activation via the mitochondrial pathway. Promoter hypermethylation with inactivation of

Apaf-1 was also shown in leukemia and correlated significantly with worse overall and disease-free survival [16]. Furthermore, Apaf-1 protein deficiency was shown in human leukemic cells and resulted in resistance to apoptosis [17]. Apaf-1 as part of the apoptosome was detected in ovarian cancer cells and demonstrated diminished activity resulting in decreased caspase activation [18].

In our study, cervical cancers with absent or weak Apaf-1 expression had significantly more lymph node metastases at time of surgery than tumors with strong or moderate Apaf-1 expression. A similar finding was reported by Baldi et al. [19] in patients with melanoma, where a borderline significant difference in Apaf-1 immunoreactivity was shown between primary melanomas that developed metastases and those that did not. In the same study, the authors found significant differences in Apaf-1 expression between primary melanomas and lymph node metastases as well as between malignant melanoma and benign nevi. The loss of Apaf-1 expression in melanoma when compared to nevi was confirmed by Dai et al. who also demonstrated that overexpression of Apaf-1 can sensitize melanoma cells to anticancer drug treatment [10].

To the best of our knowledge, this is the first report demonstrating loss of Apaf-1 as a marker of aggressive tumor behavior in cervical cancer. Future studies will have to address the question whether loss of Apaf-1 affects clinical outcome in cervical cancer patients.

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Lack of Apoptotic Protease Activating Factor-1 Expression and Resistance to Hypoxia-Induced Apoptosis in Cervical Cancer

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Abstract

Purpose: Clinical observations suggest that intratumoral hypoxia increases the aggressiveness of tumors through clonal selection of cancer cells that have lost their apoptotic potential. The aim of this study, therefore, was to investigate the expression of the proapoptotic protein apoptotic protease activating factor-1 (Apaf-1) in cervical cancers and to analyze its relation to intratumoral hypoxia and apoptosis. Furthermore, the effect of hypoxia and apoptosis on survival was examined.

Experimental Design: In 56 patients, intratumoral oxygenation measurements and subsequent needle biopsies were done. The obtained tissue was analyzed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays and by immunohistochemistry with an Apaf-1 antibody.

Results: Apaf-1 was expressed in 86% of cancers. The median apoptosis rate was 1.0%. There was no correlation between Apaf-1 expression and intratumoral hypoxia. However, Apaf-1 expression was negative in 37.5% of hypoxic cervical cancers ($pO_2 \leq 10$ mmHg) with low apoptosis rates (≤1.0%) compared with only 5.0% in nonhypoxic cancers and hypoxic cancers with high apoptosis ($P = 0.005$; Fisher's exact test). With a median follow-up period of 44 months, there was a nonsignificant trend toward worse prognosis in the hypoxic low-apoptotic group ($P = 0.08$).

Conclusions: Although Apaf-1 is expressed in the vast majority of cervical cancers, a significant proportion of tumors with low apoptosis rates despite intratumoral hypoxia showed a lack of Apaf-1 expression. This finding suggests that loss of Apaf-1 expression is a mechanism by which hypoxic cervical cancers acquire resistance to apoptosis. Thus, low Apaf-1 expression in hypoxic tumors may be an unfavorable prognostic factor.

Hypoxia is a driving force in the malignant progression of solid tumors. Hypoxic microregions have been detected in a wide variety of solid tumors, including cervical cancer, head and neck cancer, as well as soft tissue sarcomas (1–3). In previous clinical studies, our group showed that patients with hypoxic cervical cancers had a significantly worse prognosis compared with patients with better oxygenated tumors regardless of treatment modality (2). Mechanisms by which sustained tumor hypoxia may increase aggressiveness include differential regulation of gene expression (4) and clonal selection of tumor cells that have lost their apoptotic potential (5, 6).

Physiologically, hypoxia serves as a stimulus for apoptosis (7, 8). However, in a previous study, we showed the occurrence of hypoxic cervical cancers with a low fraction of apoptotic cells and showed that these tumors were highly aggressive compared with hypoxic tumors with high apoptosis rates and nonhypoxic tumors (9). The mechanisms by which hypoxia gives rise to this apoptosis resistance have only partially been elucidated. Graeber et al. (5) showed in a mouse model that hypoxic conditions select for apoptosis-resistant $p53^{-/-}$ mouse embryonic fibroblasts. One critical regulator of apoptosis under hypoxia is the apoptotic protease activating factor-1 (Apaf-1). Apaf-1 is a crucial part of the apoptosome that is assembled in response to several cellular stresses (e.g., hypoxia, DNA damage, oncogene, activation, etc.). Activation by these signals finally leads to caspase activation via the intrinsic mitochondrial pathway resulting in apoptotic cell death (10–12). Apaf-1 knockout mice showed severe defects in the apoptotic response to hypoxic stimulation (13). This finding shows that Apaf-1 is an essential component of the apoptotic response to hypoxia *in vitro*. Recently, we have shown that Apaf-1 is expressed in cervical cancer and that an absent or weak Apaf-1 expression correlates significantly with the presence of lymph node metastasis at time of surgery (14). Furthermore, Apaf-1 deficiency was shown in several malignancies, including pancreatic cancer (15), malignant melanoma (16), and

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Table 1. Patient and tumor characteristics at the time of pretherapeutic pO_2 measurements

	No. patients	Apaf-1 negative/positive	TUNEL median (range)
FIGO stage			
I	13	2/11	1.0 (0.4-3.4)
II	18	5/13	1.1 (0.4-2.5)
III	19	0/19	1.0 (0.5-1.6)
IV	6	1/5	0.95 (0.3-1.4)
Grade			
1	8	1/7	1.05 (0.4-1.8)
2	34	6/28	1.0 (0.4-3.4)
3	14	1/13	1.0 (0.3-2.5)
pT stage			
p_{T1b1}	15	3/12	0.9 (0.4-3.4)
p_{T1b2}	2	0/2	1.65 (0.4-2.9)
p_{T2b}	5	2/3	1.0 (0.8-1.4)
p_{T4}	1	0/1	0.3
NA	33		
pN stage			
N_0	19	3/16	0.9 (0.3-3.4)
N_1	4	2/2	1.0 (0.8-1.4)
NA	33		
LVSI			
L_0	21	1/20	1.0 (0.4-3.4)
L_1	35	7/28	1.0 (0.3-2.9)
Tumor oxygenation pO_2 (mmHg)*	6.9 (0.8-33.3)		
≤10	33	7/26	1.1 (0.4-2.9)
>10	23	1/22	0.9 (0.3-3.4)
Tumor diameter (mm)*	45 (17-100)		
Patient age (y)*	47 (24-79)		
Treatment modality			
Radical hysterectomy with pelvic ± paraaortic lymph node dissection	22		
Primary exenteration	1		
Radiation therapy	33		

Abbreviations: pT stage, pathologic tumor stage; pN stage, pathologic nodal status; NA, not applicable (treated by radiation therapy); LVSI, lymphovascular space involvement.
*Median (range).

leukemia, in the last of which it was associated with poor survival (17). To our knowledge, this is the first study investigating the relationship between Apaf-1 expression, intratumoral pO_2 levels, and apoptosis rates in human malignant tumors. We show that a significant proportion of cervical cancers with low apoptosis rates despite intratumoral hypoxia showed a lack of Apaf-1 expression.

Materials, Patients, and Methods

Patients, pO_2 measurement, and tissue specimens

All patients were part of a prospective clinical study evaluating the significance of intratumoral hypoxia in cervical cancer that commenced in 2001 at the Department of Gynecology at Leipzig University (18). Intratumoral oxygenation measurement was done with the Eppendorf histography system (Eppendorf, Hamburg, and Germany) according to the standard procedure described earlier (19). The procedure was done after informed written consent was obtained from each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was done pretherapeutically in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. Per track, ~30 data points were collected starting at a tissue depth of 5 mm. To confirm that the measurement was done within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of ~2 mm in diameter and 20 mm in length was taken of each measured track after the procedure. The biopsies were formalin fixed and paraffin embedded according to standard protocols followed by an evaluation by a gynecologic pathologist. Of the patients enrolled in the study between January 2001 and February 2003, sufficient material for analysis was available in 56 cases. The median pO_2 of each track was correlated to Apaf-1 expression as well as to the apoptosis rate in the corresponding biopsy (see below).

Immunohistochemical staining for Apaf-1

Immunohistochemical staining was done as described previously (14) using a polyclonal rabbit anti–Apaf-1 antibody (ProSci, Poway, CA) and the catalyzed signal amplification system from DAKO (Glostrup, Denmark). Negative controls were done by omitting the anti–Apaf-1 antibody in the primary antibody incubation.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays

Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL). Slides were treated with the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WA) according to the instructions of the manufacturer. As a positive control, DNase-treated lymph node sections were used and for negative controls, the TdT enzyme was omitted.

Evaluation of immunostaining

Evaluation of Apaf-1 immunostaining. For the assessment of cytoplasmic staining results for Apaf-1, a predefined scoring system

based on the product of staining intensity and percentage of positive tumor cells was used (20). Staining intensity was evaluated as negative (0), weak (1), moderate (2), and strong (3) and the percentage of positive tumor cells was categorized as follows: 0, 0%; 1, 1% to 10%; 2, 11% to 50%; 3, 51% to 80%; and 4, >80%. By multiplying both components, an expression score (0-12) was obtained. A section was only counted as negative when an internal control (endothelial or peritumoral inflammatory cells) was positive for Apaf-1. Evaluation of the samples was done by two independent investigators (L-C.H. and A.S.) who were blinded to the patient data. In cases of discrepant assessment, an agreement was obtained after collegial revision using a multiheaded microscope. Cases with an expression score of 0 to 2 were considered negative, whereas all specimens with a score >2 were counted as positive.

Evaluation of TUNEL assays. To assess apoptosis, cells with clear brown nuclear labeling were counted as TUNEL positive. To determine the apoptosis rate of a tumor, the number of TUNEL-positive cells per 1,000 tumor cells was expressed in percent.

Statistical analysis

The Mann-Whitney *U* test and Fisher's exact test were used for comparisons between different groups. Correlations between two variables were described by Spearman's rank correlation coefficient (r). Overall survival (OS), with deaths due to any cause as event, and relapse-free survival (RFS), with relapse and metastases as events, were analyzed by log-rank test. Three-year survival rates are presented. P values <0.05 were considered to indicate statistical significance. A retrospective power analysis was calculated for the survival statistics. Statistical analysis was done using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany), StatXact-5 (version 5.0.3), and NCSS Trial and PASS 2002.

Results

Patient characteristics and clinicopathologic features. All cervical carcinomas were clinically staged according to International Federation of Gynecologists and Obstetricians (FIGO) criteria. The median age at diagnosis was 47 years (range, 24-79 years). In 22 of the examined cases, the tumor was resected by total mesometrial resection along with pelvic/paraaortic lymph node dissection (21). In one case (FIGO IV), the tumor was treated with curative intent by laterally extended endopelvic exenteration (22). For the surgically treated patients, the tumors were additionally staged according to the pathological tumor-node-metastasis system. Thirty-three patients were treated by radiation therapy. The distribution of FIGO and tumor-node-metastasis stages is shown in Table 1. Forty-nine tumors were of squamous cell origin, six represented adenocarcinomas and one was an adenosquamous cell carcinoma.

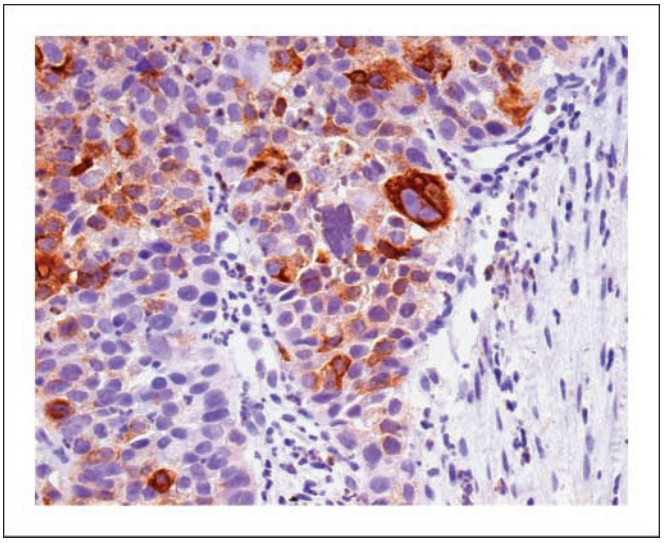

Fig. 1. Strong cytoplasmic Apaf-1 expression in a cervical cancer specimen. Magnification, ×214.

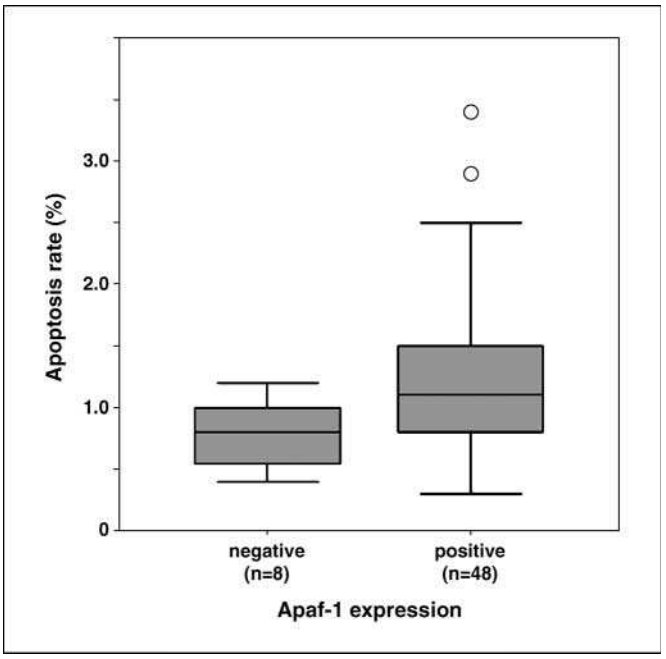

Fig. 2. Apaf-1 – positive cervical cancers have significantly higher apoptosis rates (P = 0.046). Apaf-1 expression scores of 0 to 2 are considered negative, whereas Apaf-1 expression scores of 3 to 12 are counted as positive.

Apaf-1 protein expression and apoptosis rates in cervical cancers. Immunohistochemistry for Apaf-1 was done in all 56 cervical cancer samples. Cytoplasmic Apaf-1 expression was found in 86% of the investigated cases. Positive tumor cells presented a diffuse, cytoplasmic staining (Fig. 1). There was a lower rate of Apaf-1 positivity among the FIGO I/II cases (24 of 31) compared with the FIGO III/IV cervical cancers (24 of 25). Thus, seven of the eight Apaf-1 – negative cancers belonged to the FIGO stages I and II (P = 0.063; Table 1).

The median apoptosis rate was 1.0% (range, 0.3-3.4%) as determined by TUNEL assays (Table 1). Apaf-1 – positive cancers exhibited significantly higher apoptosis rates compared with Apaf-1 – negative cases (P = 0.046, Fig. 2). This effect was even more pronounced when only regarding the group of hypoxic cervical cancers (P = 0.039).

Apaf-1 expression, apoptosis, and intratumoral pO_2. For the 56 tumors, the median oxygenation along the histologically confirmed single tracks was 6.9 mmHg (range, 0.8-33.3 mmHg). There was no correlation between Apaf-1 expression and intratumoral oxygenation (r = 0.004; P = 0.975) or between the apoptosis rates and intratumoral oxygenation (r = −0.049; P = 0.722). However, there was a group of 16 cervical cancers that exhibited low apoptosis rates despite being hypoxic (Fig. 3). To define hypoxia, the commonly used threshold of 10 mmHg was used (2) and low apoptosis was

defined as an apoptosis rate below or at the median of 1.0%. Six of the eight Apaf-1 – negative cervical cancers belonged to that group. Thus, 37.5% (95% confidence interval, 15.2-64.6%) of the cervical cancers in the hypoxic low-apoptotic group were Apaf-1 negative compared with only 5.0% (95% confidence interval, 0.61-16.9%) in the group with hypoxic high-apoptotic or nonhypoxic cervical cancers (P = 0.005, Fisher's exact test; Fig. 3).

Hypoxia, apoptosis, and survival. The median follow-up period was 44 months. For one patient, no follow-up data were available for survival analysis. Furthermore, five patients (four with disease progression and one with unknown relapse status) were not included in the analysis for RFS. There were no significant differences in OS and RFS comparing patients having hypoxic high-apoptotic or nonhypoxic tumors with patients having hypoxic low-apoptotic tumors [OS (3-year rate), 74.4% versus 50.0%; P = 0.08; power of the analysis, 45%; RFS (3-year rate), 66.3% versus 50.0%; P = 0.29]. More specifically, the comparison in OS between patients having hypoxic high-apoptotic tumors with those having hypoxic low-apoptotic cancers showed a trend toward a worse prognosis for the latter group that did not reach statistical significance [OS (3-year rate), 75.0% versus 50.0%; P = 0.18; Fig. 4]. Similarly, patients with nonhypoxic tumors showed a nonsignificant trend toward a better prognosis when compared with patients having hypoxic low-apoptotic cervical cancers [OS (3-year rate), 73.9% versus 50.0%; P = 0.12].

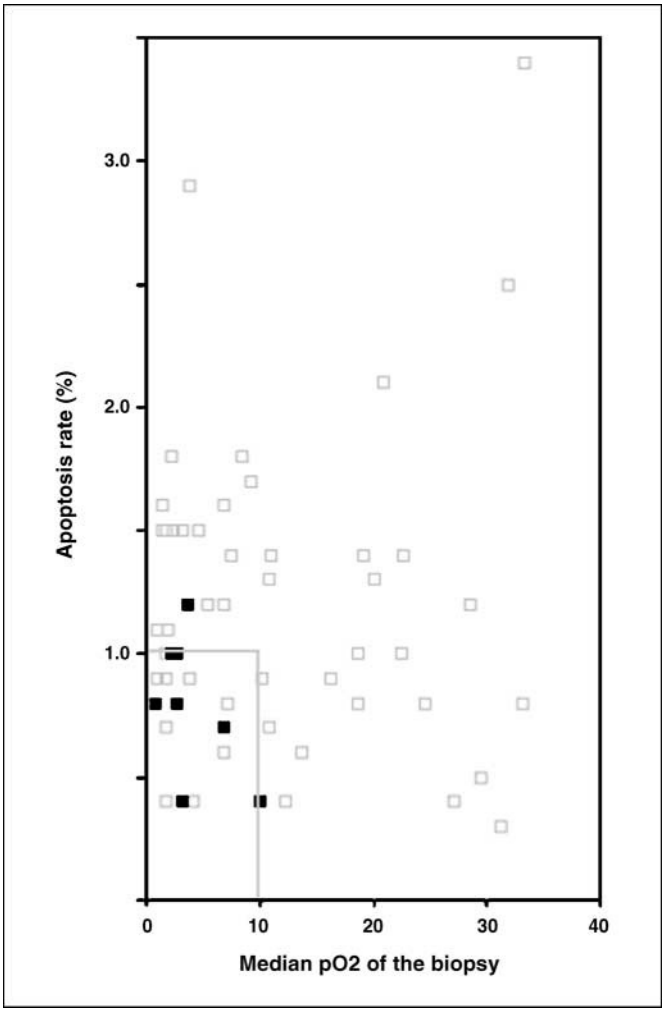

Fig. 3. Lack of correlation between the apoptosis rates (%) and the median pO_2 (mmHg) of the respective tumors. A group of 16 cervical cancers that despite hypoxia ($pO_2 \leq 10$ mmHg) exhibits low apoptosis rates (≤1.0%). Six of the eight (75%) Apaf-1 – negative cervical cancers are found in the group of hypoxic low-apoptotic tumors.

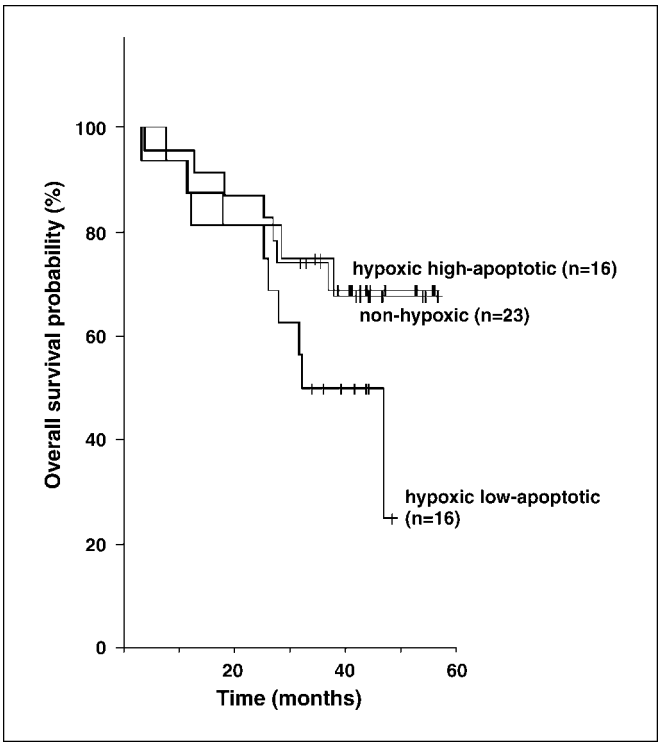

Fig. 4. Using Kaplan-Meier analysis and log-rank test, hypoxic low-apoptotic tumors showed no significant differences in OS to hypoxic high-apoptotic tumors (P = 0.18) or nonhypoxic tumors (P = 0.12), respectively.

There were no survival differences between Apaf-1 – positive and Apaf-1 – negative cervical cancers [OS (3-year rate), 65.9% versus 75.0%; P = 0.55; RFS (3-year rate), 64.5% versus 41.7%; P = 0.52].

Discussion

To our knowledge, this is the first study analyzing the relationship between Apaf-1 expression, intratumoral pO_2 levels, and apoptosis rates in cervical cancer.

Hypoxia is commonly regarded as a stimulus for apoptotic cell death (7). However, a subset of cervical cancers seems to be able to escape hypoxic induction of apoptosis. In a previous study, we found that hypoxic cervical cancers with low apoptosis rates were associated with a more aggressive phenotype resulting in poorer survival when compared with the remaining tumors (9). Likewise, in the present study, patients with hypoxic low-apoptotic tumors also had lower survival rates when compared with those with other tumors, although this trend did not reach statistical significance. As might be expected, Apaf-1 – negative cervical cancers had significantly lower apoptosis rates compared with those with Apaf-1 expression. However, this effect did not translate into a survival disadvantage for patients with Apaf-1 – negative

cancers, possibly because their tumors almost exclusively belonged to the earlier FIGO stages I and II.

The mechanisms underlying an acquired resistance to hypoxia-induced apoptosis have been addressed in *in vitro* studies and animal models (5, 6). In the present clinical study, we identified a group of 16 cervical cancers that exhibit low apoptosis rates despite intratumoral hypoxia. These 16 cervical cancers may be assumed to have grown resistant to hypoxia-induced apoptosis. One major regulator that plays a role in hypoxia-mediated apoptosis is Apaf-1. Apaf-1 knockout mice showed severe defects in the apoptotic response to hypoxic stimulation (13). We found Apaf-1 expression in 86% of our investigated cervical cancer samples. This finding is consistent with previous published data by our group that found Apaf-1 positivity in 78% of cervical cancers (14). Although the vast majority of cervical cancers express Apaf-1, 75% of the Apaf-1–negative tumors were found in the group of hypoxic low-apoptotic tumors, suggesting a mechanism by which hypoxic cervical cancers acquire resistance to apoptosis. In conclusion, one mechanism by which hypoxic cervical cancers avoid apoptosis seems to be the loss of Apaf-1 expression. Thus, low expression of Apaf-1 in hypoxic tumors may be an unfavorable prognostic factor.

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6. Abkürzungsverzeichnis

Apaf-1	Apoptotic-Protease-Activating-Factor-1
AP-1	Activating Protein-1
ATP	Adenosintriphosphat
BNIP3	Bcl-2/adenovirus E1B19kd-Interacting Protein 3
CAIX	Carboanhydrase IX
c-Met	c-Met-Protoonkogen
COX-1 / 2	Cyclooxygenase 1 / 2
DNA	Desoxyribonukleinsäure
Epo	Erythropoietin
EpoR	Erythropoietin-Rezeptor
FGF-3	Fibroblastic Growth Factor-3
FIGO	International Federation of Gynecologists and Obstetricians
GAPDH	Glyzerinaldehyd-3-Phosphat-Dehydrogenase
Glut-1 /-3	Glukosetransporter-1 /-3
HA-Tag	Hämagglutinin-Tag
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia-Inducible Factor-1
HIG1 / 2	Hypoxia-Inducible Gene 1 / 2
HNRNP	heterogenes nukleares Ribonukleoprotein
HPV	humanes Papillomavirus
IAP-2	Inhibitor of Apoptosis-2
IGFBP	Insulin-Like Growth Factor Binding Protein
iNOS	inducible Nitric Oxide Synthase
MEF	Mausembryo-Fibroblasten
MMP	Matrixmetalloproteinase
mRNA	messenger Ribonukleinsäure
NF-kB	Nuclear Factor kappa B
NIX	Nip3-like Protein X
PAI-1	Plasminogen Activator Inhibitor-1
PFK	Phosphofruktokinase
PGK	Phosphoglyceratkinase
PI(3)K	Phosphatidylinositol 3-Kinase
pO_2	Sauerstoffpartialdruck
pT	pathologisches Tumorstadium

ROS	Reactive Oxygen Species
TGF	Transforming Growth Factor
TUNEL	Terminal Deoxynucleotidyl Transferase (TdT)-Deoxyuracil Triphosphate (dUTP) Nick-End Labelling
uPAR	urokinase-type Plasminogen Activator Receptor
VHL	von-Hippel-Lindau-Tumorsuppressorgen
VEGF	Vascular Endothelial Growth Factor

7. Erklärungen

Hiermit erkläre ich, dass ich die Habilitationsordnung anerkenne.

Die vorliegende Arbeit wurde eigenständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Alles aus anderen Quellen oder von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die an der Entstehung dieser Arbeit beteiligt waren.

Ich versichere, dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke einer Habilitation oder eines anderen Prüfungsverfahrens vorgelegt wurde.

Leipzig, 20.11.2006 Dr. med. Cornelia Leo

8. Lebenslauf

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Geburtsdatum / -ort:	21. April 1973 / Leipzig
Familienstand:	verheiratet, 1 Kind

Berufliche Tätigkeit

seit 05/2000	**wissenschaftliche Mitarbeiterin an der Universitätsfrauenklinik Leipzig**
09/2004	Fachärztin für Gynäkologie und Geburtshilfe • Durchführung von Studenten- und Hebammenunterricht • Forschung zur Tumorbiologie des Zervixkarzinoms • Betreuung von 3 Doktoranden
1999 – 2000	Ärztin im Praktikum (Universitätsfrauenklinik Leipzig)
1998 – 1999	**Postdoktorandin im Department of Radiation Oncology, Stanford University Medical Center (Stanford, USA)** • molekularbiologische Forschung zu Tumor-Hypoxie und maligner Progression

Ausbildung

1998	Medizinisches Staatsexamen (Gesamtnote: 1,83)
1997	PJ-Tertial Gynäkologie am M.D. Anderson Cancer Center (Houston, USA) und an der Yale University (New Haven, USA)
1991 – 1998	Medizinstudium an der Universität Leipzig
1987 – 1991	Gymnasium Thomasschule, Leipzig Abschluß: Allgemeine Hochschulreife (Note: 1,0)

Promotion

11/1998	Institut für Biochemie, Universität Leipzig (magna cum laude) „Genotypisierung des Apolipoprotein E bei Patienten mit Demenz vom Alzheimer-Typ und Kontrollpersonen anhand von Restriktionsfragmentlängen-Polymorphismen“

Stipendium / Auszeichnungen

2005	Helga-Reifert-Preis für experimentelle Krebsforschung
1999	Bristol Myers Squibb Young Investigator Award (90th Annual Meeting of the AACR)
1991 – 1998	Studienstiftung des deutschen Volkes

Mitgliedschaften

- American Association for Cancer Research (AACR)
- Deutsche Gesellschaft für Gynäkologie und Geburtshilfe (DGGG)
- American Society for Clinical Oncology (ASCO)

Leipzig, 13.12.2006

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